CATECHOLAMINE RELEASE FROM ISOLATED CHROMAFFIN CELLS UNDER CONDITIONS OF ANOXIA OR METABOLIC INHIBITION.

by

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Declaration

The experimental work presented in this thesis was completed between October 1986 and September 1989. I declare that it is my own, and that this thesis has been composed by myself.

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Abstract

A significant release of catecholamines within the heart has been observed during myocardial ischaemia. Ischaemia-induced catecholamine release can be markedly inhibited by desipramine and other amine uptake blocking agents, allowing investigation of the importance of such release for arrhythmia production. The mechanism of this release appears to occur by a carrier-mediated efflux from neurons, which is not operative under normal conditions. The aim of the project has been to study this release process in chromaffin cells isolated from the bovine adrenal medulla, which are recognised as a model system for studying the sympathetic nervous system. Understanding this process of catecholamine release may lead to new methods of protecting the heart against ischaemia-induced arrhythmias.

Isolated chromaffin cells could be maintained in primary culture for up to 7 days and retained all their normal secretory responses. Conditions designed to mimic ischaemia, that is, anoxia or metabolic inhibition, resulted in a significant release of catecholamines. This release was shown to be independent of extracellular calcium but, in contrast to the release observed in ischaemic hearts, it was not inhibited by uptake$_1$ blockers.

One of the main criteria for exocytosis is the co-release of other secretory granule components. Polyacrylamide gel electrophoresis and Western blotting techniques were utilised to examine this following metabolic inhibition. Significant levels of the granule proteins chromogranin A, neuropeptide Y and ATP were released following metabolic inhibition, indicative of an exocytotic mechanism. Furthermore, there was no release of the cytosolic protein lactate dehydrogenase, indicating that there was no breakdown of the cell membrane during metabolic inhibition.

Over the first 10 minutes of metabolic inhibition there was a marked uptake of $^{22}Na^+$ by the cells. It is suggested that this Na^+ influx triggers the catecholamine release by affecting the cytosolic Ca^{2+} concentration through a direct effect on intracellular stores.

Intracellular Ca^{2+} mobilisation was measured using the calcium-sensitive fluorescent probe Fura-2. It was found that cytosolic free calcium levels were increased in response to metabolic inhibition.

The conditions required to evoke carrier-mediated efflux were also examined. Cytosolic levels of catecholamines could be artificially raised following treatment with reserpine. Cytoplasmic catecholamine levels were measured following permeabilisation with the detergent digitonin which renders the plasma membrane leaky. Conditions designed to reverse the uptake carrier and cause carrier-mediated efflux in the presence of raised cytoplasmic catecholamines, such as removal of extracellular sodium, did not evoke any catecholamine overflow. These studies suggest the chromaffin cell uptake$_1$ carrier is not reversible and may be gated in some way. This work has, therefore, raised questions concerning the suitability of chromaffin cells as a conventional model for sympathetic nerve terminals.

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Abbreviations

$[Ca^{2+}]_i$	cytoplasmic free calcium concentration
ABTS	2,2'-Azinobis(3-Ethylbenzthiazoline sulphonic acid)
ATP	adenosine 5'-triphosphate
ATPase	adenosine 5'-triphosphatase
Bq	Bequerels (1μCi=37Bq)
BSA	bovine serum albumin
c.p.m	counts per minute
c.p.s	counts per second
cAMP	cyclic adenosine 5'-monophosphate
CFL	Calcium free Locke's
d.p.m	disintegrations per minute
DAG	Diacyl glycerol
DBH	dopamine B hydroxylase
DMEM	Dulbeccos modified Eagles medium
DMSO	dimethyl sulphoxide
DNAse	deoxyribonuclease
DOPEG	3,4-dihydroxyphenylglycol
DTT	dithiothreitol
EGTA	1,2-di(2-aminoethoxy)ethane -N,N,N',N'-tetraacetic acid
EIPA	ethylisopropylamiloride
ELISA	enzyme-linked immunosorbent assay
Hepes	4-(2-hydroxyethyl)-1-piperazine ethane sulphonate
IP_3	inositol 1,4,5-trisphosphate
KHS	Krebs Henseleit solution
KRB	Krebs Ringer buffer
MAO	monoamine oxidase
Mes	2[N-Morpholino] ethanesulphonate
NAD	nicotinamide adenine dinucleotide
NADH	reduced nicotinamide adenine dinucleotide
PAGE	polyacrylamide gel electrophoresis
SDS	sodium dodecyl sulphate

TBS	tris buffered saline
TPA	12-O-tetradecanoylphorbol-13-acetate
Tris	tris[hydroxymethyl] aminomethane
$C_{12}E_9$	Thesit [Dodecylpoly(ethyleneglycolether)$_n$]

Contents

Chapter One

Introduction

Ischaemic heart disease is the primary cause of death in the Western world today. In England and Wales 30% of all deaths among men and 22% among women are the result of ischaemic heart disease (Dwyer and Hetzel, 1980). Improved understanding of how the heart is impaired during ischaemia and understanding of the metabolic changes that occur is vital for the development of effective prevention and treatment of the disease.

The heart is critically dependent on a constant supply of oxygen and substrate and can only tolerate an oxygen debt for a few seconds before functional changes occur. Anything that impedes oxygen availability will, therefore, have serious consequences. Myocardial ischaemia has been defined as an imbalance between the supply of oxygenated blood and the oxygen requirements of the myocardium (Reimer and Jennings, 1986). The origins of this imbalance are almost always the result of coronary artery disease, namely atherosclerosis. The formation of atherosclerotic lesions is complex and not yet fully understood. The end result, however, is the formation of plaques which occlude coronary arteries and have a tendency to fissure and rupture. Disruption of an atherosclerotic plaque leads to coronary thrombosis; that is, formation of a clot, which causes the occlusion. Following coronary occlusion the area supplied by the occluded artery becomes ischaemic since there is reduced blood flow to the myocardium and there is a rapid loss of myocardial contractility.

1.1 Biochemical consequences of myocardial ischaemia.

The underlying mechanisms by which myocardial ischaemia leads to a loss of contractile function within seconds have now been established. These include acidosis, reduced sensitivity of contractile proteins to calcium, and intracellular accumulation of inorganic phosphate and lipid, in the form of acyl CoA esters. Very rapidly after the onset of ischaemia aerobic respiration declines and there is a marked depletion of high energy phosphates (ATP and creatine phosphate). Creatine phosphate content of the myocardium falls by over 80% within 2 to 3 min, with ATP content declining more slowly (Alpert, 1989). Initially, anaerobic glycolysis is stimulated but this is subsequently inhibited by the development of acidosis and the accumulation of citrate, NADH and lactate (formed from pyruvate).

The generation of lactic acid and the conversion of oxygen to CO_2 are probably the major causes for the early development of acidosis. The mechanism by which

acidosis reduces contractility, however, is still unclear (Poole-Wilson, 1987). Contraction is normally initiated by the rapid release of calcium from the sarcoplasmic reticulum, this release itself being triggered by a rise in cytosolic calcium. Acidosis is known to affect the transport of calcium across the sarcolemma and the uptake of calcium by the sarcoplasmic reticulum. Cytosolic calcium combines with specific receptor sites on the regulatory protein, troponin, which ultimately leads to muscle tension and shortening. An increase in intracellular H^+ concentration may alter the interaction of calcium with troponin, thus impairing the actin-myosin interaction. These processes would lead to a reduction in contractility.

The second major consequence of acute myocardial ischaemia is an alteration in the form of action potentials and the subsequent development of arrhythmias. This change in action potential is a direct response to changes of pH and extracellular potassium levels. Early in ischaemia there is a marked rise in extracellular potassium due to increased efflux. This efflux is thought to be the consequence of increased conductance, intracellular accumulation of permeant anions such as lactate, and reduced activity of the Na^+/K^+ ATPase. The change in extracellular potassium and the cellular acidosis are sufficient to account for alterations in the action potential and other electrophysiological effects (Hirche *et al.*,1980). Initially this inhibition of cellular functions is reversible, but if ischaemia is of sufficient severity and duration (longer than 15 to 20 min) the myocytes become irreversibly damaged and undergo cellular necrosis, that is, the myocardium becomes infarcted.

In conjunction with these metabolic changes within the first hour after the onset of myocardial ischaemia there is a progressive release of noradrenaline from the adrenergic nerve terminals of the myocardium (Abrahamsson *et al.*,1984). This overflow of noradrenaline has long been recognised as being of central importance in the development of arrhythmias (Rona,1985). This locally-released noradrenaline has a metabolic role in the activation of anaerobic glycolysis early in ischaemia, but may also increase calcium influx to the myocyte, contributing to the progression of ischaemic cell injury and death. It is this overflow of noradrenaline along with the ischaemia-induced changes in myocyte function that lead to the development of ventricular fibrillation . In this condition there is chaotic electrical disturbance of the ventricles, ventricular contraction is uncoordinated and ventricular filling ceases. Untreated ventricular fibrillation is

invariably fatal, being the single most important cause of death in patients with ischaemic heart disease.

The mechanisms of noradrenaline release during myocardial ischaemia will be discussed here, rather than the post-synaptic events via which catecholamine stimulation exerts its effects. However, in order to understand the role of catecholamines in ischaemic heart disease it is first necessary to describe their function under normal physiological conditions.

1.2 Autonomic regulation of cardiac contractility

The sympathetic nervous system is the primary mediator influencing the contractility of the left ventricle in response to stress. Sympathetic activation increases the release of endogenous catecholamines, which results in an elevation in arterial pressure by increasing peripheral resistance, heart rate and myocardial contractility. This response is mediated by stimulation of α and β adrenergic receptors in the sarcolemma, predominantly via β_1 receptors which respond to neurally released noradrenaline. This, in turn, leads to a rise in intracellular cyclic AMP via activation of adenylate cyclase. Cyclic AMP activates cAMP-dependent protein kinases, resulting in phosphorylation of a number of intracellular proteins. This has the overall effect of increasing calcium influx by increasing the number of open channels and thus increasing contractility of the myocardium (Braunwald *et al.*,1988).

1.3 Circulating catecholamines

In contrast to neurally-released catecholamines, circulating catecholamines play little, if any, role in modulation of myocardial contractility. Catecholamines are released from the adrenal medulla into the blood stream in response to increased sympathetic nervous activity. The changes in plasma catecholamines do not, however, parallel changes in myocardial function (Young *et al.*,1985). The concentration of neurotransmitter at the nerve terminal is critical and this is not necessarily reflected by levels of circulating catecholamines.

1.4 Molecular mechanisms of nerve terminal function.

Much of the elucidation of the molecular mechanisms of synthesis, storage and secretion of catecholamines has come from studies on isolated chromaffin cells from the adrenal medulla and on isolated chromaffin granules, the secretory vesicles of the chromaffin cell. Chromaffin cells share a common embryological origin with sympathetic neurons, both being derived from the neural crest.

Furthermore, after several days in culture, chromaffin cells send out axon-like processes indicative of the neuronal character of chromaffin cells (Livett, 1984). These morphological similarities to sympathetic neurons and their relative ease of isolation have led to chromaffin cells being accepted as an ideal model for studying sympathetic nerve function. Therefore, in the following description of nerve terminal function it will be assumed that chromaffin cells are analogous to sympathetic neurons, unless otherwise mentioned, and references will be made to both systems .

1.4.1 Catecholamine synthesis.

The biosynthetic pathway of noradrenaline and, in chromaffin cells, its subsequent conversion to adrenaline, has long been established (Kirchner, 1975). Dopamine is formed from the sequential action of tyrosine hydroxylase and aromatic L-amino acid decarboxylase, enzymes which are located in the cytosol. Tyrosine hydroxylase is the rate-limiting enzyme, its catalytic activity being regulated by the availability of the substrate, tyrosine,and cofactors, and by phosphorylation at several sites. Cytosolic dopamine is then rapidly transported into secretory vesicles by an ATP-driven carrier-mediated process. Once within the vesicles dopamine is converted to noradrenaline by the enzyme dopamine β-hydroxylase (DBH). DBH is present within the vesicles in both membrane-bound and soluble forms. In chromaffin cells, the conversion of dopamine to noradrenaline within the vesicles has a $t_{1/2}$ of approximately 6h, the rate of conversion being dependent on substrate availability as discussed by Corcoran *et al.* (1984). In sympathetic neurons this is the final step in the synthetic process, while in chromaffin cells noradrenaline may be further converted to adrenaline by the cytosolic enzyme noradrenaline N-methyl transferase. Noradrenaline leaks slowly into the cytosol where it is rapidly converted to adrenaline which is transported back into the vesicles. The adrenal medulla contains two types of chromaffin cells, those which produce and store primarily adrenaline and others primarily noradrenaline.

1.4.2 Storage of catecholamines.

Catecholamines are stored in chromaffin granules at a concentration of 0.6M, less than 0.01% of total cell catecholamines being found in the cytosol (Phillips, 1982). This results in a very large concentration gradient across the granule membrane. Within the membrane of the granules is a specialised

proton-translocating ATPase that provides the driving force for the uptake of catecholamines and maintainance of their high intravesicular concentration. The ATPase hydrolyses ATP and translocates protons into the vesicles leading to the generation of an electrochemical proton gradient, composed of ΔpH (inside acidic, about pH 5.5) and $\Delta\psi$ (inside positive, about 60mV) both of which are coupled to catecholamine uptake (Apps *et al.*,1980). An uncharged amine molecule is believed to be the substrate for the amine translocator, but this is protonated on release to the interior of the granule.

Chromaffin granules also contain high concentrations of nucleotides, 0.18M, primarily ATP, which are taken up by a relatively non-specific nucleotide translocase, driven by the membrane potential. This raises the question of how such high catecholamine and ATP concentration gradients are maintained across the membrane and how spontaneous osmotic lysis of the granules is prevented. The granule membrane is relatively permeable to catecholamines which will leak out into the cytosol down their concentration gradient. This diffusion is of physiological significance in the synthesis of adrenaline. Active uptake of catecholamines into the granules is able to counteract leakage to a certain degree. Studies on artificial solutions of chromaffin granule components and intact granules have shown that catecholamines and ATP interact, probably by charge interaction and hydrophobic stacking. The consequent reduction of activities of the components reduces leakage rates and renders the granules osmotically stable (Koppell and Westhead, 1982). There is no defined stoichiometric relationship between catecholamines and ATP (Winkler and Westhead,1980). Also, the chromaffin granules are not uniform with regard to their catecholamine and ATP content so any estimates of the catecholamine/ATP molar ratio represents an average measurement. The catecholamine/ATP ratio is higher in noradrenaline-storing synaptic vesicles than in noradrenaline-containing chromaffin granules. Unlike chromaffin granules they do not contain very much ATP but have high concentrations of noradrenaline which tends to leak more rapidly from the vesicles than that in chromaffin granules (Phillips, 1982). Furthermore, their noradrenaline content increases as they mature during axoplasmic transport (Lagercrantz,1976).

1.4.3 Other components of chromaffin granules.

The major constituents of chromaffin granules are shown in Table 1.1. These will be discussed briefly.

Table 1.1 Major constituents of chromaffin granules.

Constituent	Number of molecules	
	membrane-bound	soluble
Catecholamines		3×10^6
Nucleotide		930,000
Ascorbic acid		120,000
Calcium		90,000
Chromogranin A		5,000
Chromogranin B		80
Chromogranin C		?
Dopamine β hydroxylase	210	140
Total enkephalin equivalents		4,000
Cytochrome b_{561}	1750	
Neuropeptide Y		428

Data taken from Winkler *et al.* (1986).

A. Protein constituents.

The major matrix proteins found in chromaffin granules are the chromogranins. Three groups of these acidic proteins have been identified, chromogranins A,B and C, which together comprise the majority of total soluble protein (Winkler,1988). Chromogranin alone makes up about 80% of this in bovine granules. In addition to the chromogranins, enkephalin-containing peptides and neuropeptide Y are present, comprising 1% and 0.2% of total soluble proteins respectively.

Further constituents of known function are DBH (4%) and various proteases involved in the processing of proenkephalin and other proteins. The physiological function of the chromogranins, the major secretory proteins, is still unclear. They were previously thought to play a role in the storage of catecholamines although it has now been demonstrated that there is little interaction between chromogranins and catecholamines (Winkler and Westhead, 1980). They may play some role in stabilising secretory granules and in calcium binding within the granule. More recently Simon *et al* (1988) have proposed a role for chromogranin-derived peptides in the regulation of adrenal secretion.

DBH is the enzyme responsible for the conversion of dopamine to noradrenaline within the secretory granule. It exists in two forms, a soluble form and a membrane-bound form, present in approximately equal amounts in bovine granules. The two forms of the enzyme are not identical, as suggested by their behaviour in Triton X-114 (Pryde and Phillips, 1986) and their subunit structures. The enzyme is a mixed function oxidase, requiring oxygen and ascorbate as cofactors for its catalytic activity. Ascorbate is present in chromaffin granules in high amounts, 13-22mM. It supplies two electrons to the DBH catalysed reaction, itself being oxidised to semidehydroascorbate in the process. Enzyme-bound copper acts as an intermediate electron-carrier. The hydroxylation reaction is shown below:-

$$\text{Dopamine} + O_2 + 2H^+ + 2e^- \longrightarrow \text{noradrenaline} + H_2O$$

B. Membrane constituents.

Cytochrome b_{561}, one of the most abundant proteins of the chromaffin granule membrane (15 to 19% of the total), is transmembranous. Ascorbic acid is unable to permeate the granule membrane, but cytochrome b_{561} is thought to transport

electrons from cytosolic ascorbate to intragranular semidehydroascorbate. Ascorbate is thus regenerated within the chromaffin granule and DBH activity is maintained.

Other major membrane constituents include DBH and H^+ATPase, both of which have been discussed above. Other membrane proteins and glycoproteins have no known function at present, and those responsible for transport functions of the granule remain to be characterised.

1.4.4 Catecholamine storing vesicles in sympathetic nerves.

There are two types of vesicles in sympathetic nerves, large dense core vesicles and small dense core vesicles. The large vesicles take up catecholamines and nucleotides by the same uptake mechanisms as chromaffin granules (Lagercrantz,1976). They contain DBH so are able to synthesise noradrenaline and, upon stimulation, secrete noradrenaline, ATP, chromogranins, enkephalins and neuropeptide Y. The synthesis of noradrenaline occurs at a site removed from the nerve terminal. Vesicles are formed near the Golgi apparatus and then transported down the axon to the nerve terminal where they are available for exocytosis (Reichardt and Kelly, 1983). In contrast, catecholamine synthesis and storage mechanisms in chromaffin cells takes place at the same site. Unlike the large dense core vesicles of sympathetic neurons, small dense core vesicles do not contain soluble proteins and peptides. They can,however, take up catecholamines and nucleotides. It has been suggested that they are formed from large vesicles during membrane retrieval after exocytosis and then refilled with catecholamines and ATP at a high rate near the nerve terminal rather than returning to the cell body (Winkler, 1988). Both types of vesicles secrete their contents by exocytosis and in all other aspects appear similar to chromaffin granules.

1.5 Mechanism of exocytosis in isolated chromaffin cells and sympathetic neurons.

The evidence for exocytosis as the mechanism of secretion of catecholamines was first presented by Schneider *et al.*(1967). They demonstrated that stimulation of adrenal glands with the acetylcholine analog carbachol resulted in the co-release of catecholamines and matrix proteins; the secretion of both being calcium dependent. The ratios of catecholamines and protein secreted were the same as those found in isolated chromaffin granules. These results, in conjunction with earlier reports on the co-release of catecholamines and other granule components and morphological evidence from electron microscopy, led to the conclusion that secretion from the adrenal medulla occurs by exocytosis. This term, first used by

de Duve (1963), describes the movement of a vesicle towards the plasma membrane, its fusion with it and subsequent release of its contents into the extracellular space.

Douglas *et al.* (1967) first reported that acetylcholine, the physiological secretagogue, depolarises isolated chromaffin cells by increasing the permeability of the cell membrane to certain ions which then activate secretion. It is now known that depolarisation triggers the opening of calcium channels. There is a transient increase in the cytosolic calcium concentration and a chain of events is set in motion that leads to secretion. The use of electric fields (Baker and Knight,1983) to create small holes in the plasma membrane has enabled workers to manipulate cytosolic calcium levels in order to trigger exocytosis independently of events at the plasma membrane. The use of permeabilised cells conclusively demonstrated the calcium-dependence of exocytosis and also the dependence of secretion on ATP and magnesium. Following secretion, surplus membrane is retrieved and reinternalised by the process of endocytosis. Like exocytosis, many of the mechanisms of endocytosis and vesicle recycling are yet to be elucidated.

1.5.1. Role of ion channels and membrane potential.

In the nerve terminal depolarisation of the plasma membrane is triggered by voltage-sensitive Na^+ channels. In bovine chromaffin cells interaction of acetylcholine with its nicotinic receptor causes the opening of the receptor's ion channel which allows the entry of Na^+ and Ca^{2+}. Influx of Na^+ further depolarises the cells activating the voltage-dependent Ca^{2+} channels. This allows entry of Ca^{2+} into the cell and cytosolic Ca^{2+} levels rise. The rise in cytosolic Ca^{2+} then triggers exocytosis. Catecholamine secretion can also be triggered by high extracellular K^+ concentrations. Depolarisation by high K^+ opens voltage-sensitive channels directly, bypassing the acetylcholine receptor. The contribution of these various ion channels to the secretory response depends on the concentration of secretagogue. Tetrodotoxin, which selectively blocks the fast Na^+ channels, has no effect on secretion when maximal concentrations of agonists are used. At low agonist concentrations, however, catecholamine secretion is reduced by tetrodotoxin (Kilpatrick *et al.*,1981). This suggests that, at low agonist concentrations, membrane depolarisation is partly due to nicotinic ion channels and partly due to opening of fast Na^+ channels. At high agonist concentrations, however, the resulting depolarisation is sufficient to directly open the Ca^{2+} channels without the contribution of fast Na^+ channels. Consequently, secretion is

not inhibited by tetrodotoxin or removal of extracellular Na^+. Following an action potential resting Na^+ and K^+ levels are restored by the Na^+/K^+ ATPase. The Na^+ gradient that it maintains is essential to control intracellular osmolality and is also utilised in co-transport of various substrates such as amino acids and neurotransmitters.

1.5.2. Evidence for granule and plasma membrane fusion.

There is now overwhelming evidence that secretion of catecholamines following stimulation takes place by an exocytotic mechanism. The first evidence was provided by the observations that constituents of the chromaffin granules such as the chromogranins and DBH are co-released with catecholamines. Furthermore, the release of these proteins is calcium-dependent. Immunological techniques have shown that chromogranin A and DBH are released in the same catecholamine: protein ratios as are found in isolated chromaffin granules. In addition, the cytoplasmic enzymes noradrenaline N-methyl transferase and lactate dehydrogenase are not released. Evidence for the co-release of other granule components such as neuropeptide Y, nucleotides and enkephalins has also been obtained.

Secretion of catecholamines is not accompanied by release of the granule membrane constituents, but specific membrane markers do appear on the plasma membrane. This has been demonstrated by the use of antibodies against membrane components, primarily DBH (Phillips *et al.*,1983). Stimulation of chromaffin cells with various secretagogues resulted in the exposure of DBH on the cell surface. This was visualised by immunofluorescence microscopy, using anti-DBH antibody and a fluorescent second antibody. The appearance of DBH on the cell surface was calcium-dependent and inhibited by drugs that blocked catecholamine secretion. Quantitation of the amount of DBH exposed on the plasma membrane correlated closely with the degree of catecholamine secretion (Hunter and Phillips,1989). Finally, the exocytotic event itself has been visualised using freeze-fracture techniques (Aunis *et al.*,1979) and electron microscopy which showed chromaffin granules adjacent to and fused with the plasma membrane following stimulation. It is worth noting here that the co-release of a cell component with catecholamines does not, however, imply that that component is sequestered in the same subcellular compartment as the catecholamines. For instance, the co-secretion of catecholamines and newly acquired ascorbate has been reported by Knoth *et al.* (1987). Release of the newly acquired ascorbate,

however, displays different properties to that of catecholamines suggesting they are released from different sub-cellular compartments.

Similar studies to those on chromaffin cells have established that noradrenaline secretion from sympathetic neurons is via calcium-dependent exocytosis (Winkler, 1988).

1.5.3 Role of calcium.

The absolute dependence of exocytosis on extracellular calcium has long been recognised (Douglas and Rubin, 1961). Calcium enters the cell following depolarisation through voltage-sensitive calcium channels and, in the case of nicotinic stimulation, through nicotinic receptor-linked channels. The necessity for calcium entry into the cells was conclusively demonstrated by Baker and Knight (1978). They rendered the plasma membrane of chromaffin cells leaky by exposure to a small number of high-voltage discharges. The plasma membrane is permeable to small molecules allowing direct manipulation of the cytosolic environment whilst the granules remain intact. They demonstrated that addition of calcium to permeabilised cells caused catecholamine release in a dose dependent manner. Mg^{2+}-ATP was also required for exocytosis. The development of fluorescent calcium indicators has enabled the change in intracellular calcium in response to various stimuli to be monitored directly (Knight and Kesteven, 1983; Burgoyne, 1984; Kao and Schneider, 1986; and Cobbold *et al.*,1987). These indicators, such as quin-2 and fura-2, permeate the plasma membrane as acetoxy methyl esters and enter the cytosol where they are hydrolysed to yield the impermeant indicators. This allows changes in cytosolic free calcium to be determined in the nanomolar range. Using such indicators the resting cytosolic $[Ca^{2+}]$ is found to be approximately 100nM, values ranging from 50 up to 150nM having been reported. Upon stimulation with either nicotine or high K^+ there is a transient elevation of free calcium to micromolar levels. On measurements in single cells injected with aequorin (Cobbold *et al.*, 1987) this calcium transient lasted only 60 to 90 seconds.

Fura-2 in particular has been used in the study of $[Ca^{2+}]_i$ in both cell populations and single cells. As an indicator it has many advantages over quin-2, including sensitivity (Grynkiewicz *et al.*, 1985). Recently O'Sullivan *et al.* (1989) have used video digital imaging techniques to determine the spatial distribution of $[Ca^{2+}]_i$ in resting and stimulated single cells loaded with fura-2. Video imaging

demonstrated a rise in $[Ca^{2+}]_i$ less than 2 seconds after stimulation with nicotine or high K^+ that was initially restricted to a region immediately beneath the plasma membrane. This is presumably due to the entry of Ca^{2+} via the voltage-sensitive Ca^{2+} channels. Following diffusion of the Ca^{2+} to fill the cytosolic space there was a second larger rise in $[Ca^{2+}]_i$ throughout the whole cell, due to release of Ca^{2+} from intracellular stores. Thus most of the elevation in $[Ca^{2+}]_i$ that is monitored by fluorescence measurements of cell suspensions occurs, not at the site of exocytosis adjacent to the plasma membrane, but within the cytoplasm. The release of Ca^{2+} from internal stores may be mediated by Ca^{2+} itself or by IP_3 which is produced following nicotinic or high K^+ treatment. It has been suggested that Ca^{2+} influx itself may activate phospholipase C resulting in production of IP_3 from polyphosphoinositides (Eberhard and Holz, 1987). This rise in cytosolic Ca^{2+} is now known to have numerous targets within the cell.

A Cytoskeleton.

Before exocytosis can take place it is necessary for the granule to be translocated to the plasma membrane. This has suggested a role for the cytoskeleton in granule movement and membrane fusion that may be regulated by $[Ca^{2+}]_i$. The secretory granules of unstimulated cells are trapped within a three-dimensional filamentous mesh. A ring of F-actin adjacent to the cell periphery has been observed in association with fodrin, a calcium-regulated protein that binds to and forms cross-links with F-actin. Caldesmon, a calmodulin-regulated protein which reversibly binds F-actin, is also exclusively located at the cell periphery. This high density of actin filaments and actin-binding proteins form a barrier to exocytosis at the cell periphery. Cheek and Burgoyne (1986) demonstrated that nicotine stimulation of chromaffin cells resulted in a rapid, transient disassembly of actin filaments that was consistent with the time course for catecholamine secretion, providing evidence for the involvement of the cytoskeleton in exocytosis. This disassembly was independent of extracellular calcium and unaffected by trifluoperazine, a calcium-calmodulin inhibitor. This suggests that granule movement may also be controlled by other second messengers which are calcium-independent. Other proteins associated with actin are also, however, activated by the calcium transient; for instance, caldesmon-actin cross-linking is inhibited by an increase in calcium (Burgoyne and Cheek, 1987). Thus it has been

proposed (Aunis and Bader, 1988) that the calcium transient activates proteins that break the F-actin network such as fodrin and caldesmon. However, the ability of nicotine stimulation to evoke cytoskeletal actin disassembly was not abolished in the absence of Ca^{2+}, unlike that produced by high K^{+}. This suggests that, in the case of nicotinic stimulation, mechanisms other than calcium-evoked actin disassembly must be involved in secretion (Burgoyne *et al.*, 1989). The dissolution of the cytoskeletal barrier permits release of granules from the cytoskeletal network, with consequent movement to and fusion with the plasma membrane.

A similar mechanism has been proposed in nerve terminals. Synapsin 1, a vesicle-binding protein not found in chromaffin cells, traps vesicles in the cytoskeleton by binding to the vesicle membrane and actin filaments. Depolarisation leads to a calcium-calmodulin dependent phosphorylation of synapsin 1 resulting in detachment of the secretory vesicles from the cytoskeleton, De Camilli and Greengard (1986).

B Calcium-binding proteins.

In addition to calmodulin, a number of proteins have been described that bind reversibly to chromaffin granule membranes in a calcium-dependent manner (Geisow and Burgoyne, 1982). Calmodulin binds to chromaffin granules and facilitates the binding of cytosolic proteins to granules. Furthermore, injection of chromaffin cells with anti-calmodulin antibodies inhibited catecholamine secretion (Kenigsburg and Trifaró, 1985). When cytosolic proteins that have leaked out of digitonin-permeabilised chromaffin cells are added back, exocytosis is restored in the presence of calcium (Sarafian *et al.*, 1987). One of these proteins leaked from the cells was identified as calmodulin. Calmodulin alone, however, cannot restore the secretory response suggesting a role for other cytosolic proteins.

Several other proteins have been identified that reversibly bind to secretory vesicle membranes in the presence of calcium, including a family of closely related proteins, the annexins (Burgoyne and Geisow, 1989). One member of this family, calpactin, has been identified as having a possible role in exocytosis. Calpactin is localised at the inner surface of the plasma membrane and binds to fodrin and actin in a calcium-dependent manner. Recently, Ali *et al.* (1989) demonstrated that calpactin was able to restore the secretory response of permeabilised chromaffin cells, which had been lost due to leakage of cytoplasmic proteins. This evidence

indicates that calpactin, at least, is essential for exocytosis. Other calcium-binding proteins have been identified but whether or not they play a role in exocytosis has yet to be established.

C Role of MgATP.

Baker and Knight (1981) in their work on electrically permeabilised chromaffin cells demonstrated that exocytosis requires not only calcium but also exogenous MgATP. Readdition of either magnesium or ATP alone to electro -permeabilised cells does not trigger exocytosis, both being required. Pretreatment of cells with metabolic inhibitors which deplete cytoplasmic pools of ATP abolishes the response of both intact and digitonin-permeabilised cells to secretagogues or direct calcium challenge (Nakanishi *et al.*, 1988). Millimolar levels of MgATP are required for exocytosis. Several roles have been suggested for MgATP in exocytosis. For example, maintenance of the granule membrane electrochemical gradient requires metabolic energy, but Knight and Baker (1985) demonstrated that inhibition of the H^+-translocating ATPase or dissipation of the granule membrane potential or pH gradient does not affect exocytosis. A number of proteins have been identified that are phosphorylated in a calcium-dependent manner (Geisow and Burgoyne, 1987) but whether they are involved in exocytosis is not known. The only phosphoprotein known to be involved in exocytosis is Synapsin 1 and that is found in nerve terminals but not in chromaffin cells (see section A Cytoskeleton).

The observations that certain phorbol esters can enhance the sensitivity of exocytosis to calcium and that this is blocked by inhibitors of protein kinase C have suggested the involvement of protein kinase C in exocytosis (Knight *et al.*, 1988; and Burgoyne *et al.*, 1988). The sensitivity of secretion and protein kinase C to the same inhibitors suggests that the latter is involved in the actual mechanism of exocytosis rather than its modulation. This is supported by the findings that protein kinase C is translocated to membranes in response to nicotine stimulation or high K^+ within seconds (Terbush *et al.*, 1988). This leads to increased protein phosphorylation. Protein kinase C has a specific requirement for MgATP within the concentration range required for secretion. The targets of this enzyme, however, still remain unknown and MgATP may be involved in processes other than phosphorylation.

1.5.4. Calcium removal from the cytoplasm.

As has been discussed above, the cytosolic rise in $[Ca^{2+}]_i$ that triggers secretion is transitory, $[Ca^{2+}]_i$ returning to basal levels within 1 minute following nicotine or high K^+ treatment in chromaffin cells (Cobbold *et al.*, 1987). Therefore specific mechanisms must exist to remove calcium and maintain basal levels in the absence of any stimulus. Chromaffin granules have an internal calcium concentration of approximately 20mM. Only about 0.03% of this is present as free ions, the remainder being bound to acidic components inside the granules such as the chromogranins and the interior of the granule membrane (Haigh *et al.*, 1989). As the granule calcium reserve constitutes the majority of total cell calcium this suggests that granules may be important in removal of calcium from the cytoplasm following a calcium transient. Calcium is taken up into the granules via a Na^+/Ca^{2+} exchange system linked to a Na^+/H^+ antiporter, described by Haigh and Phillips (1989). The Na^+ gradient set up by the antiporter can act as an energy source to drive Ca^{2+} transport. Von Grafenstein and Powis (1989) have recently suggested that the release of calcium from chromaffin granules by exocytosis may be important in long term calcium homeostasis since ultimately calcium must be removed from the cell.

Kao (1988) studied the uptake of $^{45}Ca^{2+}$ into intracellular organelles following stimulation of digitonin-treated chromaffin cells. In the short term the amount of calcium taken up by the intracellular organelles accounted for only 5 to 10% of that influx. Most of this calcium was taken up in an ATP-dependent manner into the endoplasmic reticulum. Uptake by the mitochondria only became significant when $[Ca^{2+}]_i$ reached micromolar levels. Thus it seems that small fluctuations in calcium levels during resting states are probably controlled by the endoplasmic reticulum. Following depolarisation the bulk of the cytosolic calcium must be immediately extruded from the cell via the calcium pump and Na^+/Ca^{2+} exchange system of the plasma membrane.

Similar mechanisms for removal of calcium from the cytoplasm have been identified in nerve terminals; these being efflux via Na^+/Ca^{2+} exchange and Ca^{2+}-dependent ATPase and, to a lesser extent, sequestration in intracellular organelles (Reichardt and Kelly, 1983).

1.5.5. Inactivation of released catecholamines.

There are two mechanisms by which the action of noradrenaline at the sympathetic

nerve terminal and catecholamines in the circulation is terminated.

A Re-uptake.

The neuronal uptake system, uptake$_1$, represents the major mechanism for terminating the action of noradrenaline at the sympathetic nerve terminal. Uptake$_1$ has been well characterised in neurons (Paton, 1976). PC12 cells, a clonal cell line from rat phaeochromocytoma, have been used as a model system for adrenergic neurons to characterise its molecular mechanism (Bönisch *et al.*,1984).

Noradrenaline is taken up via a sodium- and chloride-dependent symporter. Sodium forms a Na^+-carrier complex at the side of the plasma membrane where [Na^+] is high (and [K^+] low), that is, on the outside under normal physiological conditions. Uptake is energy-dependent since it is driven by an inwardly-directed Na^+ gradient, maintained by the Na^+/K^+ ATPase. Both Na^+ and Cl^- facilitate the binding of noradrenaline. When noradrenaline is bound the complex becomes mobile and noradrenaline is transported together with its co-substrates Na^+ and Cl^-. Once inside, the high intracellular K^+ concentration competes with Na^+ for a common binding site on the complex causing Na^+ to dissociate. K^+ replaces Na^+ in the carrier-complex and is transported out in the direction of the K^+ gradient. Noradrenaline transport is thus electroneutral, one Na^+ being exchanged for one K^+. At the outside of the membrane where [Na^+] is high K^+ is dissociated from the carrier and the Na^+-carrier complex formed fixes substrate binding sites at the outer face of the membrane. The energy dependence of uptake$_1$ has been demonstrated using metabolic inhibitors that rapidly deplete cellular ATP thus dissipating the Na^+ and K^+ gradients across the plasma membrane. Uptake$_1$ is inhibited by drugs such as cocaine and tricyclic antidepressants. The dependence of uptake$_1$ on an inwardly directed Na^+ gradient is demonstrated by replacement of extracellular Na^+ by other monovalent cations; this results in efflux of noradrenaline from the axoplasm of adrenergic nerve terminals (Paton, 1973). This efflux is mediated by reversal of the Na^+-dependent uptake^1 carrier. The physiological significance of this "carrier-mediated efflux" will be discussed in the next section.

A high affinity uptake system for catecholamines has also been characterised in chromaffin cells (Kenigsburg and Trifaró, 1980; and Banerjee *et al.*, 1987).

This uptake system possesses similar saturation kinetics and has an absolute requirement for extracellular Na^+. Uptake is dependent on maintenance of an inwardly directed Na^+ gradient by Na^+/K^+ ATPase and is also sensitive to inhibition by cocaine and tricyclic antidepressants.

B Metabolism.

Cytosolic catecholamines comprise less than 0.01% of total chromaffin cell catecholamines. The cytosolic catecholamine concentration is maintained at this low level by rapid uptake into secretory granules, as discussed above, and metabolism. Following re-uptake into sympathetic nerve terminals, noradrenaline can be metabolised by monoamine oxidase. This is located on the outer mitochondrial membrane and catalyses the oxidative deamination of amines. The reaction catalysed by MAO, shown below, involves the dehydrogenation of the amine at the carbon-nitrogen bond to form the corresponding imine, which is then hydrolysed to give the aldehyde plus ammonia. Oxygen is essential to act as the hydrogen acceptor.

$$RCH_2NH_2 + O_2 + H_2O \longrightarrow RCHO + NH_3 + H_2O_2$$

At least two forms of the enzyme exist, MAO type A and MAO type B. These can be distinguished by their substrate specificity and sensitivity to selective inhibitors (Youdim *et al.*, 1988). The substrate specificity of the two types is reflected in their localisation. In adrenergic nerve endings the major form of MAO is type A where noradrenaline deamination is needed for regulation of neurotransmission. In contrast, chromaffin cells possess primarily MAO type B (Youdim *et al.*, 1986) which has a low affinity for noradrenaline as the physiological function of these cells is to conserve and store catecholamines (Youdim *et al.*, 1984). Catecholamines can be methylated by catechol-O-methyl transferase prior to deamination. Catechol-O-methyl transferase is associated with an additional, extraneuronal, uptake mechanism ($uptake_2$) that is found in smooth muscle, myocardial and gland cells.

1.6 Catecholamine release during myocardial ischaemia and reperfusion.

Catecholamines are released during myocardial ischaemia and reperfusion, and may be the primary mediators of ventricular arrhythmias. Following myocardial infarction, plasma and urinary catecholamines levels are raised in man (Vettner *et al.*, 1974). Chronic cardiac denervation which depletes myocardial catecholamine stores and chemical sympathectomy using 6-hydroxy dopamine

both prevent arrhythmias during ischaemia and reperfusion (Ebert *et al.*, 1970). Such observations, although indirect, suggest that increased catecholamines released in response to myocardial ischaemia are of fundamental importance in arrhythmogenesis. Raised plasma catecholamine could come from two sources: the adrenal medulla and peripheral nerve terminals; or from the sympathetic nerve terminals within the ischaemic myocardium.

1.6.1. Peripherally-released catecholamines.

Experiments on anaesthetised rats *in vivo* (Daugherty *et al.*, 1986) demonstrated raised plasma catecholamines during acute myocardial ischaemia. There was a relationship between catecholamine levels and the subsequent development of ventricular arrhythmias. Adrenal demedullation or adrenalectomy abolished the elevation of plasma catecholamine, but had no significant effect on the development of arrhythmias. It was concluded that circulating catecholamines are not responsible for triggering arrhythmias during myocardial ischaemia.

1.6.2. Locally-released catecholamines.

The release of catecholamines within the ischaemic myocardium, either spontaneously or in response to increased sympathetic activity, is controversial. There are two main areas of dispute. There is the question of whether catecholamines released are arrhythmogenic; and secondly whether they are released into the extracellular space during ischaemia and then washed out during reperfusion or are released only as a direct result of reperfusion. The very nature of ischaemia has made it extremely difficult to separate the two events since, in ischaemia, flow to the heart is very low or completely absent. Metabolites released during ischaemia can only be measured in the venous effluent once flow is returned to normal, that is, during reperfusion. Reperfusion itself, however, exacerbates ischaemic myocardial injury and some metabolites will be released at this stage rather than during the ischaemic event *per se*.

A Arrhythmogenic nature of locally-released catecholamines.

Penny (1984) reported that chronic cardiac denervation, which depletes myocardial catecholamine stores, prevented arrhythmias during ischaemia and reperfusion. Both chemical sympathectomy using 6-hydroxy dopamine and β adrenoceptor blockade prevent arrhythmias. Similarly, Daugherty *et al.* (1986) reported a reduction in the severity of ventricular arrhythmias during ischaemia following depletion of catecholamines from the sympathetic nerve terminals in rats *in vivo*. To investigate the mechanism of this effect more closely these

authors examined the release of [^{3}H] noradrenaline in isolated perfused rat hearts following coronary artery ligation. The onset of ventricular arrhythmias was similar to that in rats *in vivo* but no enhanced release of [^{3}H] noradrenaline was seen; however, the release of [^{3}H] noradrenaline may not be the same as the release of endogenous catecholamines *in vivo*.

Riemersma and Forfar (1982) studied endogenous noradrenaline release in the venous effluent from the ischaemic myocardium in dog *in vivo*. There was a 30% incidence of ventricular fibrillation within the first 10 minutes of coronary occlusion, but no concomitant noradrenaline release. An absence of noradrenaline, however, does not necessarily imply that noradrenaline is not released at the nerve terminals in the ischaemic myocardium. There is evidence of noradrenaline loss from the nerve terminals during *in vivo* ischaemia using fluorescence techniques (Muntz *et al.*, 1984). Ischaemia was associated with a diffusion of fluorescence away from the nerve terminal that remained within the ischaemic zone suggesting that noradrenaline is released within the ischaemic myocardium but remains trapped within this zone. Furthermore, early in ischaemia, cardiac cAMP is raised and glycolysis activated, both effects mediated by catecholamines. Thus, β adrenergic antagonists such as propranolol are antiarrhythmic due to prevention of stimulation of adenylate cyclase and consequently decreased formation of cAMP (Opie *et al.*, 1979). Within the first 10 minutes of ischaemia there is enhanced sympathetic activity leading to release of noradrenaline into the extracellular space as shown by Schwartz and Stone (1980). At this time, however, there may also be increased re-uptake and α-adrenoceptor modulation at the nerve terminal accounting for the lack of noradrenaline in the venous effluent. Blockade of re-uptake with desipramine and of α_2-mediated negative feedback with yohimbine revealed a significant noradrenaline release from dog ischaemic myocardium within the first 10 minutes of coronary occlusion (Forfar *et al.*, 1985). The ligation of the coronary artery to induce ischaemia was shown to enhance sympathetic nerve activity, but even so there was no detectable noradrenaline release in the myocardial venous effluent, presumably due to enhanced re-uptake at the nerve terminal.

The absence of noradrenaline release during the first 10 minutes of ischaemia is also reported by Daugherty *et al.* (1986) who showed no elevation of catecholamines in rats *in vivo* or in perfused rat hearts *in vitro*. In contrast,

however, Hirche *et al.* (1980) reported an increase in noradrenaline from the myocardium within the first 10 minutes after the onset of coronary occlusion in pigs *in vivo*. This was associated with an early phase of arrhythmias. The ischaemic model used by Hirche *et al.* (1980), however, was one of regional ischaemia and it is possible that this noradrenaline came from normally perfused regions of the heart. Furthermore, Wilde *et al.* (1988) have recently reported that increased catecholamine release in the venous effluent is only observed after ischaemic periods longer than 10 minutes in the isolated globally ischaemic rat heart. This is in agreement with the findings of Schömig *et al.* (1984) and Carlsson (1988) and it can be concluded that there is no significant release of noradrenaline during the first 10 minutes of ischaemia.

The evidence for enhanced overflow during prolonged ischaemia is more controversial. Schömig *et al.* (1984); Abrahamsson *et al.* (1984); Carlsson (1988); and Knopf *et al.* (1988) have all reported a marked overflow of noradrenaline in the venous effluent following 15 to 60 minutes ischaemia in the isolated perfused rat heart, while *in vivo* studies in other species have suggested overflow does not occur. For instance, Forfar *et al.* (1985) reported an absence of catecholamine overflow following ischaemic periods of up to 75 minutes in dogs. In contrast, there have been consistent reports of enhanced overflow during subsequent reperfusion, (for example, Riemersma and Forfar ,1982).

Interpretation of the results from *in vivo* studies are complex as it is not clear whether overflow occurs during reperfusion or during previous ischaemia. This is discussed in detail in the next section. Despite overwhelming evidence in favour of ischaemic noradrenaline overflow in the isolated perfused rat heart, release during myocardial ischaemia *in vivo* remains controversial and observations made in isolated systems may not necessarily reflect the *in vivo* ischaemic event.

B Catecholamine overflow during ischaemia and reperfusion.

As discussed above, Riemersma and Forfar (1982) reported no overflow of noradrenaline during the first ten minutes of ischaemia, but significant overflow following reperfusion. This study, however, used a model of regional ischaemia where it is not known what the relative contributions of the ischaemic and non-ischaemic regions are in modulating levels of noradrenaline in the venous effluent. Schömig *et al.* (1984) attempted to solve this problem using isolated perfused rat hearts that were made globally ischaemic by complete abolition of coronary flow. In this way the duration and extent of ischaemia could be precisely

defined. Hearts were subject to "stop-flow" ischaemia for varying lengths of time and the catecholamine overflow measured in the venous effluent during the first 5 minutes reperfusion. Ischaemic periods greater than 10 minutes resulted in a progressive increase of noradrenaline overflow upon reperfusion. Furthermore, most of the noradrenaline was detected after only 1 to 2 minutes reperfusion suggesting that noradrenaline was released into the extracellular space during ischaemia and then washed out upon reperfusion rather than being released by the action of reperfusion itself.

The relative contributions of ischaemia and reperfusion to noradrenaline overflow cannot be completely separated by such a model. After 60 minutes stop-flow ischaemia Abrahamsson *et al.* (1983) reported a marked overflow of noradrenaline in the perfusate of isolated rat hearts. In order to examine the possibility that overflow was induced by reperfusion, hearts were reperfused with a calcium-free buffer (Abrahamsson *et al.*, 1984). A major part of the cellular damage caused by reperfusion is calcium-dependent, so it was suggested that removal of calcium during reperfusion would significantly reduce noradrenaline overflow if induced by reperfusion but have no affect if overflow occurred during ischaemia. Calcium-free perfusion had no affect on noradrenaline overflow following ischaemia suggesting that the overflow is ischaemia-induced. Dart *et al.* (1987) developed experimental models that were not dependent on stop-flow ischaemia followed by reperfusion in order to distinguish ischaemia-induced from reperfusion-induced noradrenaline overflow. The model used was one of anoxia whereby hearts were perfused at normal flow with oxygen-free and substrate-free buffer. The flow to the myocardium remains normal so any noradrenaline overflow will be in direct response to anoxia rather than reperfusion. After 15 to 20 minutes anoxia there was a marked overflow of noradrenaline of a similar order of magnitude to that seen during reperfusion following stop-flow ischaemia. In conclusion, therefore, less than 10 minutes ischaemia is not associated with significant noradrenaline release but with more prolonged ischaemia there is a marked noradrenaline overflow that is associated with the ischaemic episode rather than with reperfusion.

1.6.3 Mechanism of noradrenaline release during myocardial ischaemia.

A Early myocardial ischaemia.

In the normal myocardium adrenergic stimulation causes an increase in noradrenaline release and subsequent adrenergic stimulation of the myocytes. During the first 10 minutes of ischaemia, however, when increased sympathetic activity would be expected, there is little, if any, accumulation of noradrenaline in the extracellular space (Dart *et al.*, 1984a). Released noradrenaline is, however, rapidly removed by uptake$_1$. Blockade of uptake$_1$ with desipramine increased noradrenaline overflow during early ischaemia as shown by Forfar *et al.* (1985). In addition, stimulation-induced exocytotic release during ischaemia is modulated through presynaptic receptors (Dart *et al.*, 1984b; Schömig, 1988), and inhibition of α_2 adrenergic receptors with yohimbine and A_1 adenosine receptors with 8-phenyltheophilline enhances release. However, even with combined inhibition of uptake$_1$ and presynaptic modulation, exocytotic release of noradrenaline is still significantly lower than that released by sympathetic stimulation under conditions of normal flow. This suggests that early in ischaemia there is a failure of stimulation-evoked noradrenaline release. Exocytosis is an ATP-dependent process and ATP is probably depleted in neurons very rapidly after the onset of ischaemia. Thus early ischaemia results in a loss of sympathetic activity and after 20 minutes there is no release of noradrenaline in response to sympathetic stimulation (Dart *et al.*, 1984a). Therefore, during more prolonged ischaemia noradrenaline release is unlikely to result from enhanced neural sympathetic activity.

B Prolonged myocardial ischaemia.

Within 40 minutes after the onset of total ischaemia in the perfused rat heart more than 30% of total noradrenaline content of the heart can be recovered during subsequent reperfusion. If this noradrenaline were equally distributed throughout the extracellular space during the ischaemic period the concentration of noradrenaline is predicted to reach micromolar levels, sufficient to cause myocardial necrosis, even in the non-ischaemic myocardium. When catecholamines exceed physiological doses the energy reserve of the myocytes is depleted leading to complex biochemical and structural changes and ultimately necrosis. The crucial event in the catecholamine-induced myocardial necrosis is Ca^{2+} overload. This results in myofilament overstimulation, increase of contractile force and oxygen requirement, and increased ATP breakdown, all of which contribute to myocyte injury. In addition, several other mechanisms may

contribute to myocardial necrosis induced by catecholamines including mobilisation of free fatty acids; increased intracellular acidity; free radical production; activation of phospholipases or proteases; and catabolite accumulation (Rona, 1985).

The nature of this noradrenaline overflow was studied in detail by Schömig *et al.* (1984). There are three possible mechanisms by which this noradrenaline may be released.

1. Exocytotic release could be triggered either by increased sympathetic activity or by membrane depolarisation by extracellular potassium, which is known to reach depolarising concentrations during prolonged ischaemia. In each case release would be dependent on influx of extracellular Ca^{2+}.
2. There may be increased leakage of noradrenaline from the nerve terminals by passive diffusion. This process is unlikely to occur, however, unless there is breakdown of cell membrane structure. This only occurs after ischaemic periods greater than 60 minutes duration (Ganote *et al.*, 1976).
3. A mechanism of carrier-mediated efflux of catecholamines from the cytoplasm into the extracellular space may operate. It was postulated by Paton (1976) that under certain conditions the $uptake_1$ carrier may operate in reverse.

Carrier-mediated efflux can be characterised by its sensitivity to $uptake_1$ blocking drugs such as desipramine and cocaine, and its independence of extracellular calcium and presynaptic modulation.

Schömig *et al.* (1984) demonstrated a noradrenaline overflow following 40 minutes stop-flow ischaemia that was inhibited by more than 80% by 100nM desipramine. A similar effect was found with cocaine, nisoxetine and (+) oxaprotiline, all $uptake_1$ inhibitors. These drugs all inhibited noradrenaline overflow with the same concentration dependence as that required to block $uptake_1$. In contrast, ischaemia-induced noradrenaline release within the first 10 minutes was enhanced by $uptake_1$ blocking drugs. Agents that inhibit presynaptic modulation had no effect on this late ischaemia-induced noradrenaline overflow, unlike early release which was enhanced (Riemersma and Forfar, 1982).

Perfusion with calcium-free buffer had no effect on the cumulative overflow of noradrenaline after 20 minutes of ischaemia, even when 1mM EGTA was included in the perfusate.

From this evidence it is suggested that there are three phases of noradrenaline release during myocardial ischaemia. During the first 10 minutes (in the perfused rat heart) noradrenaline is released in response to increased sympathetic activity by an exocytotic mechanism that is calcium-dependent and sensitive to presynaptic modulation. Noradrenaline levels remain low in the extracellular space due to rapid re-uptake via neuronal uptake$_1$. The second phase of ischaemia, 15 to 40 minutes, is characterised by a progressive overflow of noradrenaline that is calcium-independent and blocked by uptake$_1$ inhibitors. It is not modulated by α_2 antagonists or electrical stimulation. Since exocytosis has an absolute requirement for calcium and ATP this cannot be the mechanism of release. Passive diffusion of catecholamines from the nerve terminal would not be sensitive to uptake$_1$ blockade. All the evidence thus points to this efflux being catalysed by the uptake$_1$ carrier, working in reverse. Finally, ischaemic periods longer than 60 minutes result in further spontaneous overflow of noradrenaline that is not blocked by uptake$_1$ inhibitors and is the result of progressive, irreversible cell injury.

The main prerequisite for the carrier-mediated efflux postulated above for the second phase of release would be a raised cytosolic catecholamine concentration. Under normal conditions this is kept low by the action of MAO, which is oxygen dependent, and the vesicular uptake of catecholamines which is dependent on an intact proton gradient across the vesicle membrane. During ischaemia it is postulated that this would be dissipated due to a rundown of ATP and cytoplasmic acidosis causing uncontrolled leakage of catecholamines into the cytoplasm. MAO will be ineffective due to a lack of oxygen so cytoplasmic catecholamine concentrations will remain high. In conjunction with changes in the transmembrane sodium and potassium gradients catecholamine efflux would occur via reversal of the uptake$_1$ carrier.

1.7 Mechanism and metabolic requirements of ischaemia- and anoxia-induced carrier-mediated efflux.

Dart *et al.* (1987) studied the metabolic conditions necessary to evoke carrier-mediated efflux, measuring noradrenaline overflow from the heart without changes in flow rate. In this way they were able to investigate the effects

of modifying oxygen and substrate supply in the absence of the secondary effects of low-flow ischaemia, such as alterations in metabolites and ion concentrations in the extracellular space. Isolated rat hearts were made anoxic by perfusion with nitrogen-saturated substrate-free perfusate. The noradrenaline overflow that ensued had the same characteristics as that produced by total ischaemia, being independent of calcium and inhibited by uptake$_1$ blockade. If glucose was present in the anoxic perfusate, noradrenaline overflow was almost completely abolished. Schömig *et al.* (1987) used three models of energy depletion to investigate this further.

1. Total ischaemia.

A significant overflow of noradrenaline was observed only after ischaemic periods greater than 10 minutes. If hearts were preperfused with substrate-free normoxic buffer for 45 minutes, or glycolysis was blocked for 5 minutes prior to ischaemia, this time lag was removed.

2. Anoxia.

Perfusion with substrate-free anoxic buffer produced a similar noradrenaline overflow that reached a peak after 40 minutes and then declined, presumably due to depletion of noradrenaline stores. The amount of noradrenaline released was far in excess of that released during ischaemia raised by cessation of flow, probably due to the continuous washout of noradrenaline in the anoxic model. During ischaemia accumulation of noradrenaline in the extracellular space would reduce the noradrenaline concentration gradient between the intracellular and extracellular spaces. In other respects anoxia-induced overflow was the same as that produced by ischaemia.

3. Cyanide intoxication.

The effects of anoxia could be mimicked using cyanide intoxication. Under these conditions it was also possible to measure DOPEG. This is a major metabolite produced by the oxidative deamination of noradrenaline, catalysed by MAO. DOPEG is lipophilic and so, unlike noradrenaline, freely diffuses out of the nerve ending. Cyanide intoxication, in the absence of glucose, mimics anoxia by inhibiting ATP production whilst MAO remains functional as oxygen is still present. Thus DOPEG can serve as an indicator of cytoplasmic noradrenaline concentrations under these conditions. Noradrenaline overflow was preceded by a rise in DOPEG concentrations; this remained high in the presence of desipramine, when

noradrenaline overflow was inhibited.

These experiments indicate that the energy status of the sympathetic nerve terminal must be the main determinant of non-exocytotic noradrenaline overflow. Both inhibition of oxidative phosphorylation and the inhibition or exhaustion of anaerobic glycolysis are required for noradrenaline overflow to occur. Complete abolition of overflow in the presence of glucose during anoxia or cyanide intoxication demonstrated that other changes that occur during ischaemia, such as accumulation of metabolites and acidosis must be of secondary importance as in models of ongoing flow they are present to a much lower degree. The parallel overflow of DOPEG in the cyanide model provides evidence that noradrenaline must enter the cytoplasm prior to efflux and further confirms a non-exocytotic mechanism of release. If noradrenaline were released exocytotically there would be no release of DOPEG since noradrenaline would not come into contact with MAO, located on the outer surface of the mitochondrial membrane.

The importance of intracellular and extracellular ion concentrations, and of Na^+/K^+-ATPase activity in noradrenaline release from the ischaemic or anoxic myocardium has also been considered (Schömig *et al.*, 1988; and Dart and Riemersma, 1989). Pharmacological agents such as the monovalent ionophore monensin and the vesicular amine-carrier inhibitor reserpine were used to disrupt the ability of the secretory vesicles to maintain a high concentration of catecholamines. In the presence of such agents cytoplasmic levels of noradrenaline were elevated, as indicated by raised levels of the metabolite DOPEG. This increased concentration of cytoplasmic noradrenaline alone was not sufficient to cause carrier-mediated efflux. However, when combined with conditions designed to cause alteration of the transmembrane sodium gradient there was a significant efflux of noradrenaline. The transmembrane sodium gradient was reversed by inhibiting Na^+/K^+-ATPase with ouabain, by opening voltage-sensitive Na^+ channels with veratridine, or by replacing extracellular sodium with sucrose. Alteration of the sodium gradient alone did not trigger efflux. Thus both elevated cytoplasmic noradrenaline concentrations and an outwardly directed sodium gradient are required for carrier-mediated efflux to occur.

These metabolic interventions have enabled a scheme of events that would lead to noradrenaline overflow during myocardial ischaemia to be postulated. The rapid depletion of energy stores is central in causing the metabolic changes required for carrier-mediated efflux to occur. A rundown of ATP will lead to inhibition of the

vesicular H^+-ATPase. The transmembrane H^+ electrochemical gradient will tend to dissipate leading to accumulation of catecholamines in the cytoplasm. Under ischaemic conditions MAO is inhibited so metabolism of these catecholamines is prevented. At the plasma membrane Na^+/K^+-ATPase activity is blocked due to ATP rundown. Furthermore, intracellular acidosis activates the Na^+/H^+ exchanger, which plays a critical role in the regulation of intracellular pH (Lazdunski *et al.*, 1985). These two effects lead to intracellular sodium accumulation which facilitates catecholamine efflux via the $uptake_1$ carrier.

Thus there is good evidence to suggest that, in the absence of sympathetic nervous activity, carrier-mediated efflux will occur. The role of the $uptake_1$ carrier during myocardial ischaemia is a complex one. In regions where blood supply is maintained $uptake_1$ will operate to prevent extracellular noradrenaline levels rising. In regions where blood flow is inadequate severe ischaemia will ensue and carrier-mediated noradrenaline efflux occur. Within the myocardium the severity of ischaemia will vary, resulting in a heterogeneous overflow of noradrenaline which, in itself, will further contribute to the development of arrhythmias. All these studies, however, have been carried out on whole hearts, and the changes that are predicted to occur at the nerve terminal can only be inferred from observations made after the ischaemic event. Furthermore, all measurements of metabolites related to energy metabolism that are made in the ischaemic heart relate to the myocardium and not to the nervous system itself, since sympathetic nerve terminals make up only a tiny fraction of the total mass of the tissue. In order to overcome these difficulties there is a need for isolated cell models in which to obtain direct measurements of the metabolic changes leading to carrier-mediated efflux.

There is much evidence to suggest the use of chromaffin cells in such studies. As discussed, chromaffin cells are closely analogous to sympathetic nerve terminals and have the advantage of being readily available as a homogeneous population. Furthermore, much of what is known about sympathetic nerve terminals has been inferred from studies on isolated chromaffin cells so it would seem likely that the latter would also behave in the same manner under pathophysiological conditions. There are several lines of evidence to suggest that this may be the case.

1.8.1 Isolated sympathetic neurons.

Sweadner (1985) studied the mechanism of ouabain-evoked release of [^{3}H]

noradrenaline from primary cultures of sympathetic neurons from the superior cervical ganglion of newborn rats. Inhibition of Na^+/K^+-ATPase with ouabain resulted in release of [^{3}H] noradrenaline. Ouabain caused an efflux of approximately 50% of the total pool of [^{3}H] noradrenaline within 1 hour. This release was dependent on extracellular Na^+ but not extracellular Ca^{2+} and was inhibited by desipramine. This suggests that inhibition of Na^+/K^+-ATPase alone, in sympathetic neurons in vitro, is sufficient to cause carrier-mediated efflux of [^{3}H] noradrenaline. Ouabain may, however, have pharmacological effects other than inhibition of Na^+/K^+-ATPase, and efflux of [^{3}H] noradrenaline may not necessarily reflect the efflux of endogenous catecholamines under the same conditions.

1.8.2 Cortical synaptosomes.

Of more relevance to the overflow of noradrenaline in the ischaemic myocardium is the release of glutamate that occurs during anoxia in isolated rat cerebral cortical synaptosomes (Kauppinen and Nicholls, 1986; Kauppinen *et al.*, 1988; and Sánchez-Prieto and González, 1988).

Anoxia in the brain is known to cause a large release of glutamate which may be responsible for the irreversible damage to neurons that occurs under these conditions. Two pools of glutamate were identified in brain synaptosomes. The first, a vesicular pool, can be released by calcium- and energy-dependent exocytosis. However, as in the ischaemic myocardium, this exocytotic release fails within the first 5 minutes after cyanide treatment of isolated synaptosomes. The second glutamate pool is cytoplasmic and can be released via reversal of the Na^+-dependent co-transporter in a Ca^{2+}-independent manner. This Ca^{2+}-independent release is activated during anoxia and becomes the major mode of glutamate efflux. In conjunction with carrier-mediated glutamate efflux there is a parallel decline in ATP, demonstrating the importance of energy depletion in triggering efflux.

Whilst there are many similarities between anoxia-induced glutamate release in synaptosomes and ischaemia-induced noradrenaline overflow in the myocardium, it should be noted that the bulk of synaptosomal glutamate is found in the cytoplasm rather than being sequestered within vesicles. Efflux of glutamate is observed within the first 5 minutes after the onset of anoxia and may well occur spontaneously under normoxic conditions as vesicular depletion of glutamate or rundown of energy stores are not prerequisite for efflux. In contrast, significant

noradrenaline overflow is not observed for at least 10 to 15 minutes after the onset of total ischaemia. Before this time anaerobic glycolysis is sufficient to maintain energy-dependent processes and prevent elevation of intra and extracellular noradrenaline levels.

1.8.3 PC12 cells.

PC12 cells are a clonal cell line obtained from a rat phaeochromocytoma, first isolated by Greene and Rein (1977). Most PC12 cell lines express the same properties as adrenal chromaffin cells, such as high levels of catecholamine-synthesising enzymes and high endogenous concentrations of catecholamines. As in chromaffin cells, catecholamines are sequestered in reserpine-sensitive granules. catecholamines can be released by a Ca^{2+}-dependent mechanism upon exposure to nicotine or high K^+. Greene and Rein (1977) characterised a transport system for noradrenaline uptake by PC12 cells that was energy- and sodium-dependent, and was sensitive to cocaine and desipramine. It was concluded that this was similar to neuronal $uptake_1$.

The $uptake_1$ system of PC12 cells has since been extensively characterised (Bönisch *et al.*, 1984). When PC12 cells were loaded with [^{3}H] noradrenaline in the presence of reserpine, to inhibit vesicular uptake, and pargyline, to inhibit MAO, the label accumulated in the cytoplasm. On exposure to agents that cause an influx of sodium, such as veratridine, or to a sodium-free medium, carrier-mediated efflux of [^{3}H] noradrenaline was observed. This efflux showed similar properties to that observed in ischaemic hearts in that it was calcium-independent and sensitive to blockade by cocaine and desipramine. The observation that carrier-mediated efflux occurs in PC12 cells suggests that, under the right conditions, such a reversal of the $uptake_1$ carrier would also occur in chromaffin cells. It should be noted that observations on PC12 cells relate solely to [^{3}H] noradrenaline, and not to efflux of endogenous catecholamines, and this efflux was only observed in response to artificially imposed conditions. There are no reports on the effects of anoxia on catecholamine efflux in PC12 cells.

1.8.4 Adrenal glands *in vivo* and *in vitro*.

While there are no reports of carrier reversal in chromaffin cells there is indirect evidence to suggest that such a system, as described in PC12 cells, will also operate. Bülbring *et al.* (1948) studied the secretion of adrenaline from

perfused dog adrenal glands. When perfused with nitrogen-saturated blood there was a significant overflow of adrenaline within 30 minutes. A similar effect was demonstrated by injection with potassium cyanide which produced an immediate increase in adrenaline output.

More recently, hypoxia during birth has been associated with a large release of catecholamines from the adrenal medulla in humans. These catecholamines are essential in protecting the neonate from the harmful effects of hypoxia, and in survival outside the womb (Lagercrantz and Slotkin, 1986). In neonatal rats the release of adrenal catecholamines caused by hypoxia occurs independently of sympathetic stimulation since splanchnic control of adrenomedullary function is absent at birth (Slotkin and Seidler, 1988). This suggests that some alternative mechanism, in direct response to the hypoxic state, is responsible for the release of adrenomedullary catecholamines during birth.

1.9 Outline of project.

It was proposed to use isolated bovine adrenal chromaffin cells as a model for sympathetic nerve terminals to study the mechanism of catecholamine release under "ischaemic" conditions. Non-exocytotic release can be identified by its calcium-independence and sensitivity to uptake$_1$ blockers. Although carrier-mediated efflux has been demonstrated in isolated sympathetic neurons and PC12 cells, it has not been demonstrated under conditions designed to simulate myocardial ischaemia. Pilot experiments carried out in this laboratory suggested that such a release does occur. Chromaffin cells made anoxic with oxygen-free, substrate-free buffer released catecholamines within the first hour. This release was reduced in the presence of desipramine. It was proposed to use isolated chromaffin cells to establish the exact metabolic conditions required to evoke catecholamine release, that is, the dependence of release on oxygen deprivation and lack of glycolytic substrates. The cellular events leading up to efflux, such as release from granules to cytosol, could then be investigated.

Energy depletion of the cells under anoxic conditions could be monitored by measuring cytosolic ATP levels, following the bioluminescence of the firefly luciferase/luciferin reaction. It is possible to gain direct access to the cytosol by permeabilisation of chromaffin cells with digitonin, a process that leaves granule membranes intact. This method would also allow direct measurement of other cytosolic parameters such as Na^+ and catecholamines. The Na^+-dependence of

carrier-mediated efflux could also be investigated not only by altering extracellular Na^+ concentrations but also by monitoring the routes of Na^+ influx under anoxic conditions with $^{22}Na^+$. Similarly, fluorescent indicators could be utilised to measure changes in intracellular pH and calcium levels. It was hoped that detailed understanding of the mechanism of catecholamine release might suggest new methods of preventing such release which would be effective under ischaemic conditions but would not interfere with normal adrenergic regulation of cardiovascular function.

During the development of this project it was observed that the release of catecholamines from chromaffin cells evoked by "ischaemic" conditions did not share the same characteristics as carrier-mediated efflux. Furthermore, this release was found to be distinct from normal exocytotic release evoked by nicotine or high K^+. Therefore, this project developed into an investigation of the mechanism of this "ischaemia"-induced release and a reassessment of the observations in ischaemic hearts that led to the mechanism of carrier-mediated efflux being postulated. It has also raised important questions concerning the suitability of isolated chromaffin cells as a model for sympathetic nerve terminals.

Chapter Two

Materials and Methods

2.1 Materials

All chemicals were of analytical grade or the highest chemical purity available and were supplied by the Sigma and BDH Chemical Companies, both of Poole, Dorset, U.K. All radiochemicals were obtained from Amersham International, Amersham, Bucks, U.K. Scintillation fluid was obtained from LKB, Loughborough, Leicestershire, U.K.

Cell culture materials were obtained from Gibco-Ltd, Paisley, Scotland. Luciferin and luciferase were obtained from Boehringer Mannheim GmbH, Biochemica, West Germany. Fura-2/AM was obtained from Novabiochem (U.K) Ltd, Nottingham, England.

Ethylisopropylamiloride was kindly supplied by Prof. F. Lang, Institut Für Physiologie, University of Innsbruck, Austria.

Samples of (+) and (-) oxaprotiline were a gift from Dr. J. Stephens, Institute of Psychiatry, London, England.

Calcium-free Locke's solution
154mM NaCl; 5.6mM KCl; 5.5mM glucose; 5.0mM Hepes; 3.6mM $NaHCO_3$; 5 i.u/ml penicillin; and 5μg/ml streptomycin; pH 7.4.

Calcium-free Krebs Ringer buffer
145mM NaCl; 5.0mM KCl; 1.0mM NaH_2PO_4; 10mM glucose; and 20mM Hepes; pH 7.4.

Locke's solution
154mM NaCl; 5.6mM KCl; 5.5mM glucose; 5mM Hepes; 2.2mM $CaCl_2$; and 1.2mM $MgSO_4$; pH 7.4.

ATP assay buffer
154mM NaCl; 2.6mM KCl; 2.15mM K_2HPO_4; 0.85mM KH_2PO_4; 2.2mM $CaCl_2$; 1.18mM $MgSO_4$; and 10mM glucose; pH 7.4.

Veronal buffer
50mM Na barbitone; 85mM NaCl; 0.5% (w/v) BSA; and 0.01% (w/v) NaN_3; pH8.0.

Horse serum buffer
2.5% (w/v) BSA; 0.5mg/ml NaN_3; 5% (v/v) inactivated horse serum; 20mM Tris-HCl; and 0.9% (w/v) NaCl; pH 7.2.

Krebs-Henseleit solution
144.14mM NaCl; 4.02mM KCl; 1.85mM $CaCl_2$; 1.05mM $MgSO_4$; and 10mM glucose; pH 7.4; and 26mM $NaHCO_3$.

Digitonin permeabilisation buffer
- Ca^{2+} buffer:139mM potassium glutamate; 20mM PIPES; 5mM MgATP; 5mM glucose and 5mM EGTA, pH 6.6.

Methods

2.2 Isolation and maintenance of bovine adrenal medullary chromaffin cells .

Two methods have been employed to isolate chromaffin cells.

Method 1

Chromaffin cells were isolated from bovine adrenal glands by a modification of the method of Wilson and Viveros (1981). All solutions used were obtained sterile or were prepared in deionised water and sterilised by filtration through 0.22μm filters. All glassware was sterilised by autoclaving. Bovine adrenal glands were obtained from the local slaughterhouse as soon as possible after slaughter of the animal. Glands were transported to the laboratory encased in fat, in calcium-free Locke's solution (CFL). All glands collected were trimmed of fat and only those that were a healthy pink colour in appearance with no obvious deformities were used. Three to four glands were generally selected for further processing. These were fine-trimmed of fat leaving a "collar" round the adrenal vein for cannulation. The adrenal vein was cannulated with plastic tubing attached to a 20ml syringe containing CFL prewarmed to 37°C. Any leakages were stopped with ligatures. Multiple slits, 1mm in depth, were made in the cortex of the gland to allow perfusion of the buffer through the gland. The cannulated glands were then attached to a multichannel peristaltic pump, and the glands perfused retrogradely with CFL at 37°C for 20min at a flow rate of 5 to 10ml/min, to wash out red blood cells. Perfusion of the glands was then changed to CFL containing 0.1% (w/v) collagenase; 0.5% (w/v) bovine serum albumin (BSA); and 15μg/ml deoxyribonuclease for 15 to 30min. Perfusion time was adjusted depending on the specific activity of the collagenase preparation. The glands were washed free of collagenase by perfusion with CFL for a further 5 to 10min. The medullae were then removed by dissection and finely minced in a small volume of CFL under sterile conditions. The mince was further digested for 10min, 37°C, with fresh collagenase solution with intermittent stirring. The crude cells were filtered through 250μm nylon mesh and collected by centrifugation for 10min at 1000rpm (85xg). Pellets were resuspended in CFL, washed and filtered through 85μm nylon mesh a further two times. Cells were resuspended in DMEM, 37°C, after the final wash and recentrifugation. Resuspended cells were mixed with Percoll (14ml cells per 12.6ml Percoll), the latter prepared by mixing 9 vol of Percoll with 1 vol of 10x CFL and adjusting to pH 7.4. Each Percoll gradient took

approximately $100x10^6$ cells. The cell suspensions were placed in sterile polycarbonate centrifuge tubes with caps and centrifuged at 15000rpm (20000xg) for 20min at 20°C in a Beckman rotor (50.2Ti type). The chromaffin cell fraction was collected and washed three times in DMEM by centrifugation at 1000rpm (85xg) for 10min. Finally the cells were resuspended in DMEM prior to plating.

Method 2

The second method used to isolate chromaffin cells was modified from that of Knight and Baker (1983). Four adrenal glands collected and trimmed of fat, as described above, were injected via the adrenal vein with 3x10ml calcium-free Krebs Ringer buffer (KRB) to wash out red blood cells and tissue debris. Each gland was then injected with 2x5ml 0.1% (w/v) protease, incubating the glands for 15min, 37°C, after each injection. Medullae were then removed by dissection and minced in KRB under sterile conditions. The minced medullae were further digested with 0.1% (w/v) collagenase and 15µg/ml deoxyribonuclease, in a tissue culture flask, made up to a final vol of 10ml plus 10ml per gland with KRB. After gassing the mince for 2min with 95% O_2/5% CO_2 the flask was vigorously shaken at room temp for 20 to 30min. The resulting cell suspension was filtered through muslin prior to filtering through 250µm nylon mesh. All subsequent washing and purification steps were carried out as described in Method 1.

2.2.1 Cell viability

Cell viability and yield were determined by diluting 50µl cell suspension with 50µl trypan blue (0.04% (w/v) in phosphate buffered saline). Trypan blue exclusion indicated cell viability as observed using phase contrast microscopy. Usually 90 to 95% of cells isolated by these methods are viable.

2.2.2 Culture conditions

Cells were diluted to the required density with plating medium consisting of 50% DMEM and 50% Ham's F-12 nutrient mix with 5mM Hepes and 2.438g/l $NaHCO_3$ (pH 7.4 at 37°C and 5% CO_2). This was supplemented with 4% (v/v) foetal calf serum; 10i.u/ml penicillin; 10µg/ml streptomycin; 10µg/ml gentamycin; 25µg/ml fluorodeoxyuridine; and 0.25µg/ml fungizone. Just before use 3µg/ml cytosine arabinoside and 50µg/ml ascorbate (pH 7.4) were added to the medium. Cells were plated at a density of $0.5x10^6$ cells/ml in 24-well tissue culture

plates, 1ml/well, unless otherwise stated. Cell cultures were maintained at 37°C in a humidified atmosphere containing 5% CO_2. Cultured cells were utilised after a minimum of 2 days in culture. If utilised after 4 to 5 days in culture plating medium was replaced with fresh medium minus foetal calf serum and cytosine arabinoside, supplemented with fresh ascorbate.

2.3 Catecholamine secretion

Chromaffin cells cultured in multiwell plates were utilised for secretion experiments. Plating medium was replaced with 1ml Locke's solution with or without secretagogue. After 10min at 20°C the supernatant was removed and cells were lysed by addition of 1ml Locke's solution plus 1% (w/v) Triton X-100. Both cell supernatants and cell lysates were assayed for catecholamine content immediately, or frozen and assayed at a later date.

2.4 [^{3}H] Noradrenaline labelling of chromaffin cells.

Chromaffin cells in multiwell dishes were incubated overnight at 37°C with 0.3ml DMEM containing 1μCi(37Bq)/0.3ml d,l [^{3}H] noradrenaline, specific activity 8 to 15Ci (296 to 555Bq)/mmol and 0.01% (w/v) ascorbic acid (Kilpatrick *et al.*, 1980). The cells were then washed 2x by incubating in 1ml DMEM for 15min at 37°C. Total uptake of [^{3}H] noradrenaline by the cells was determined by lysing the cells with 0.3ml Locke's solution plus 1% (w/v) Triton X-100 and counting aliquots in a liquid scintillation counter. Alternatively, when [^{3}H] labelled cells were used to study secretion, experiments were initiated by replacing the DMEM with 0.6ml Locke's solution with or without secretagogues. After time the supernatant was removed and the radioactivity remaining in each well determined by adding 0.3ml Locke's solution plus 1% (w/v) Triton X-100. Aliquots of supernatant and cell lysates were then counted for radioactivity (see section 2.5).

2.5 Uptake of $^{22}Na^+$ by chromaffin cells.

Medium was aspirated from chromaffin cells in multiwell dishes and replaced with Locke's solution, 250μl/well, containing 2.6μCi (96.2Bq)/ml ^{22}NaCl. After time 100μl aliquots were removed for catecholamine determination. The remainder was aspirated and wells washed 3x with Locke's solution within 10sec. Cells were lysed with Locke's solution plus 1% (w/v) Triton X-100, 350μl/well, and 50μl aliquots taken for catecholamine determination or measurement of $^{22}Na^+$ by scintillation counting. $^{22}Na^+$ uptake was calculated

from the initial specific activity of the Locke's solution and expressed as nmol of $^{22}Na^{+}$ taken up/well.

2.6 Scintillation counting

Radioactive samples were mixed with 2ml Optiphase 'Safe', a multi-purpose liquid scintillation cocktail. Cpm were measured for 2min and automatically corrected for quenching to give dpm. External standards of known radioactivity were also counted for calibration. All samples were treated in the same way.

2.7 Assay Methods

2.7.1 Catecholamine assay

Catecholamines were assayed fluorimetrically by the trihydroxyindole method of von Euler and Lishajko (1961). Cell supernatants or lysates were adjusted to pH 6.2 with 0.01M potassium phosphate buffer (pH6.2) in a final vol of 1ml. Oxidation of catecholamines was carried out for 3min by addition of 0.1ml 0.25% (w/v) potassium ferricyanide. The strongly fluorescent trihydroxyindole formed by the addition of alkali was stabilised by including ascorbic acid and 1,2 diaminoethane in the mixture. Two ml of this alkali-ascorbate were added (9vol 4M NaOH: 1vol 2% (w/v) ascorbic acid: 0.2vol 1,2 diaminoethane). Fluorescence was measured after 15min and within 2h at 2 sets of excitation/emission wavelengths 395/490nm and 436/540nm using a Perkin Elmer 300 fluorescence spectrophotometer. Calibration was carried out by comparison with known adrenaline and noradrenaline standards which fluoresce at different intensities at the 2 sets of wavelengths.

Catecholamine content was calculated as follows:

Aa - fluorescence of 1nmol adrenaline at a)

Ab - fluorescence of 1nmol adrenaline at b)

Na - fluorescence of 1nmol noradrenaline at a)

Nb - fluorescence of 1nmol noradrenaline at b)

where a) = 395/490nm and b) = 436/540nm.

$$\text{Noradrenaline (nmol)} = y = \frac{(M\ Ab/Aa) - N}{(Na\ Ab/Aa) - Nb}$$

Where M = fluorescence at 395/490nm and N = Fluorescence at 436/540nm

$$\text{Adrenaline (nmol)} = x = \frac{N - y\ Nb}{Ab}$$

Results were corrected to give catecholamine content (nmol/ml) and expressed as

a percentage of the total catecholamine content of the cells. All release experiments were carried out on triplicate wells.

This method enabled a differential estimation of noradrenaline and adrenaline to be made by measuring the excitation-emission of a single sample at two different wavelengths. Fluorescence readings were found to increase for the first 15min but were then stable for at least 2h, allowing a large number of samples to be processed at any one time. Like other available methods for determining catecholamines, such as HPLC or radioenzymatic assay, this method is only as accurate as the standards used. There is a linear relationship between fluorescence intensity and catecholamine concentration over the range found in chromaffin cells (Fig. 2.1). Standards were always assayed in triplicate, dilutions being made up fresh each time and the concentration adjusted by measuring the absorbance of a 1/100 dilution at 280nm. The stock (10mM) was then diluted accordingly to give a working concentration of 1mM. The reproducibility of the assay was assessed by comparing the fluorescence readings of adrenaline and noradrenaline standards at the two sets of wavelengths from 30 different assays (Table 2.1). The coefficient of variation was calculated for these standards and was less than 10% in each case. This gives an indication of the reliability of the assay as a reproducible method for measuring catecholamines.

Table 2.1

Differential estimation of noradrenaline and adrenaline.

	Aa	Ab	Na	Nb
mean ± SE	15.4 ± 0.24	14.0 ± 0.24	25.3 ± 0.42	4.5 ± 0.1

Values are the absolute fluorescence means ± SE of 30 estimates of 1nmol adrenaline or noradrenaline measured at 395/490nm (Aa and Na) and 436/540nm (Ab and Nb).

2.7.2 Lactate dehydrogenase assay

Lactate dehydrogenase was measured by monitoring the formation of NADH from NAD at 340nm as described by Kelner *et al.* (1986). Aliquots (100µl of cell supernatant or lysate) were microfuged at high speed for 5min then incubated with 60mM lactate/100mM Tris, pH 9.0 and 1mM NAD in a final vol of 1.1ml at 20°C for 30min.

2.7.3 Protein estimation by dye binding

The method used was that described by Bradford (1976). The dye binding assay

used gave an absorbance change of 0.25/5μg of BSA and was linear between 2 to 8μg. The concentration of BSA was calculated from its absorbance at 280nm.

2.7.4 ATP assay method

ATP was assayed by monitoring the bioluminescence of the firefly luciferase/luciferin system, (White *et al.*, 1987; Rojas *et al.*, 1985). Highly purified luciferase and luciferin were used. Luciferase was made up in 1ml ice-cold Tris-acetate buffer, 0.5M, pH 7.5, to give a luciferase concentration of 1mg/ml. This could be stored frozen. When required aliquots were diluted with assay buffer plus 0.5% (w/v) BSA to give a working concentration of 200μg/ml. Luciferin was made up fresh each time in assay buffer to a final concentration of 1.25mg/ml. Luciferin solutions were stable for up to 48h when stored at 4°C. ATP (disodium salt) for calibration was made up in 0.01M potassium phosphate buffer containing 4mM magnesium sulphate, pH 7.4. Concentrations were quantitated spectrophotometrically at 259nm ($\Sigma^{10\mu M} = 0.154$). Serial dilutions of the stock solution were made in the potassium phosphate buffer. The assay was performed as follows: equal volumes of luciferase and luciferin were mixed prior to use; 20μl ATP standard of known concentration was added to 180μl assay buffer in a glass scintillation vial; 10μl luciferase/luciferin mixture was added and bioluminescence monitored over 3min in a Packard 3330 Tri-carb scintillation spectrometer, with coincidence counting. Mean cpm were plotted on a log-log scale to give a standard curve that was linear between 10^{-6}M and 10^{-8}M ATP, (Stanley and Williams, 1969) as shown in Fig 2.2. The light intensity was essentially constant for at least 10min in response to low concentrations of ATP. The enzyme concentration used was kept to a minimum so that the rate of ATP consumption was low enough to prevent the ATP concentration changing during the reaction.

2.7.5 On-line detection of ATP release from isolated chromaffin cells in suspension.

Freshly isolated chromaffin cells were plated in bacteriological petri dishes at a density of 0.5×10^6 cells/ml, in plating medium as previously described. Cells treated in this way do not attach to the plates but remain in suspension. 20μl of cell suspension (approximately 1000 to 2000 cells) was added to 180μl assay buffer in the presence or absence of secretagogues, plus 10μl luciferin/luciferase mixture. Light output was monitored over time as described. At the end of each assay total ATP content of the cells was determined by addition of 20μl assay buffer plus 1% (w/v) Triton X-100. The amount of ATP present was calculated

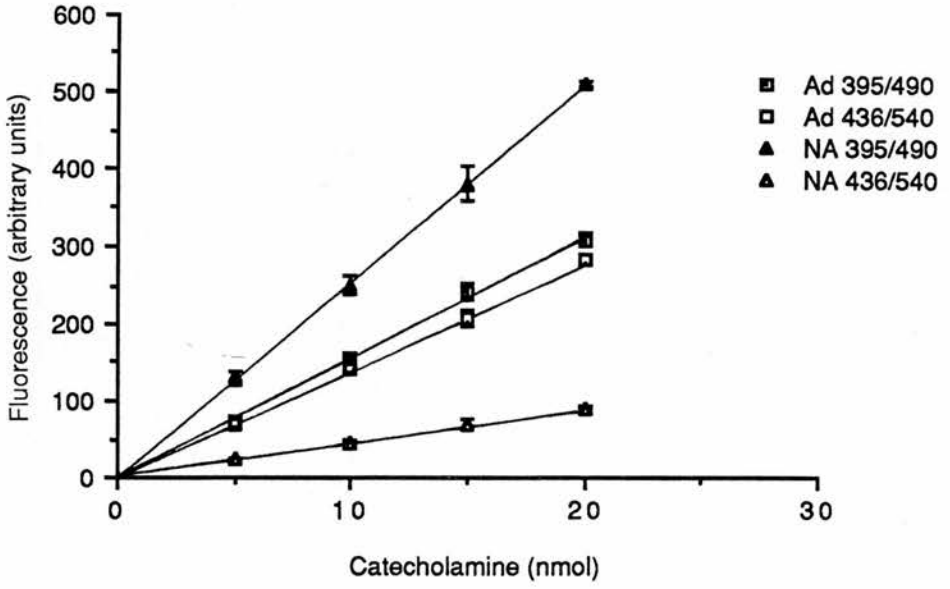


Fig 2.1 Catecholamine standard curve. Fluorescence of known standards of adrenaline and noradrenaline was measured at two different emission-excitation wavelengths. The results shown are means ± SE of triplicate determinations.

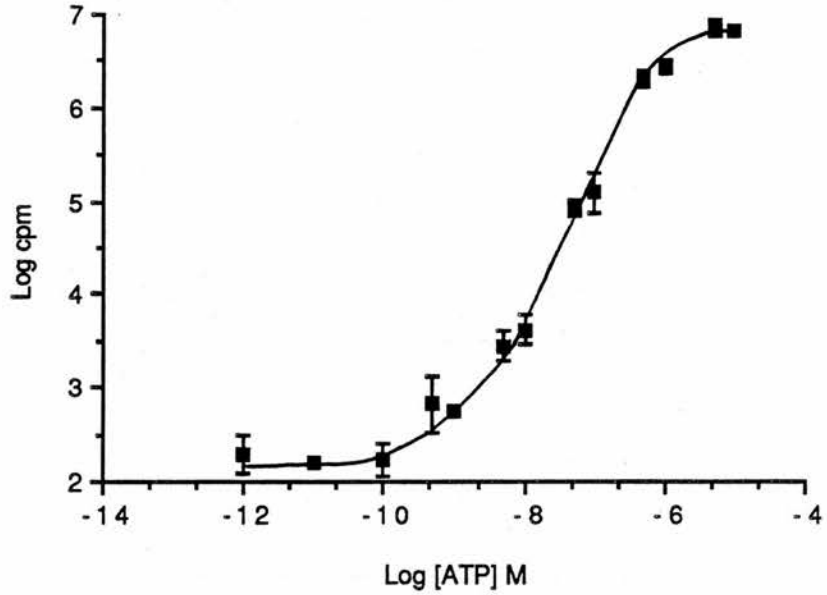


Fig 2.2 ATP standard curve. The light intensity of a range of ATP concentrations was recorded and plotted as log cpm versus log [ATP] M, means ± SE of triplicate determinations.

from the standard curve (Fig 2.2) the system being calibrated after every assay (see Chapter 6).

2.7.6 Detection of granule marker proteins using ELISA.

Ninety-six well Falcon ELISA plates were coated with poly-L-lysine (20mg/ml in 10mM Mes/Tris buffer, pH 6.5) for 1h at 37°C. The antigen was added to coated plates, 100µl/well and incubated for 90min at 37°C. If necessary the antigen was diluted in 10mM Mes/Tris buffer, pH 6.5. The antigen used comprised either chromaffin granule lysate or supernatants from chromaffin cells subjected to a variety of conditions. Excess buffer was removed by washing 4x with Veronal buffer. After washing wells were blocked by incubation with 100µl/well Veronal buffer for 1h, 4°C. First antibody (diluted 1 in 100 in horse serum buffer) was added and wells incubated for 90min, 37°C. After washing 4x with Tris-buffered saline (TBS) plus 0.1% (v/v) Tween 20 wells were treated with second antibody anti-rabbit IgG-biotin (diluted 1 in 1000 in TBS plus 5% (v/v) sheep serum) for 2h, 37°C. After further washing plates were incubated with streptavidin-peroxidase (diluted 1 in 1000 in TBS) for 1h. A final washing took place prior to development of the plates. To develop 100µl ABTS (0.55mg/ml in phosphate/citrate buffer) plus 0.002% (v/v) H_2O_2 was added to the wells and incubated for 10min, 37°C. Colour development was stopped with 0.1M citric acid. Colour intensity was quantitated by measuring the absorbance of each well at 410nm using a Dynatech Microplate reader.

2.7.7 Determination of neuropeptide Y

Endogenous neuropeptide Y was determined by radioimmunoassay as described by Richardt *et al.* (1988). RIA was carried out on cell supernatants following 10min treatment with Locke's buffer alone or plus 10µM nicotine or metabolic inhibitors. Neuropeptide Y determination was carried out by Dr. M. Haass, Dept. of Cardiology, University of Heidelberg, Heidelberg, Federal Republic of Germany, and this assistance is gratefully acknowledged.

2.7.8 Cortisol determination

Cortisol was measured in fractions taken from Percoll gradients by radioimmunoassay by Mr. E. Lightly, Dept. of Clinical Chemistry, Edinburgh Royal Infirmary, Edinburgh, Scotland.

2.8 Detection of chromaffin granule proteins by PAGE and Western blotting.

Proteins were separated on 10% acrylamide gels in the presence of SDS, (Douglas

and Butow, 1973). The discontinuous buffer system of Laemmli and Favre (1973) was used. After separation on polyacrylamide gels proteins were electrophoretically transferred to nitrocellulose for decoration with antibodies. Cell supernatants and granule lysate were microfuged at high speed for 2min to remove any cell debris and then diluted 3:1 in sample buffer plus 0.001%(w/v) bromophenol blue and 10μM DTT. The protein content of granule lysate was determined by Bradford assay and then diluted accordingly to give a predicted protein content of 0.05 to 0.4μg (that predicted to be present in the cell supernatants).

2.8.1 Immunoblotting.

A horizontal electroblotting apparatus was used to transfer proteins from polyacrylamide gels to nitrocellulose sheets using wetted filter paper as the only buffer reservoir as described by Kyhse-Anderson (1984). Electrophoretic transfer took 1h at a constant current of 0.8mA/cm^2. The nitrocellulose was blocked by washing for 1h in TBS plus 0.5% (v/v) Tween 20, (Batteiger *et al.*,1982); this buffer was also used for all subsequent washing steps. The blot was then incubated with first antibody (diluted 1 in 100 in horse serum buffer) for 90min, 20°C. Following four washes the blots were incubated for 2h, 20°C, with anti-rabbit IgG-biotin (diluted 1 in 1000 in TBS plus 5% (v/v) sheep serum). Finally blots were decorated with ^{125}I-streptavidin (5cps/μl) for 1h and washed a further 4x. After drying autoradiography was carried out at -70°C. Scanning of autoradiographs using a Joyce-Loebl chromoscan 3 and measurement of peak integrals gave a numerical value for band intensity.

2.9 Overflow of noradrenaline from electrically-stimulated and ischaemic rat hearts.

Perfusion of hearts *in situ* was carried out as described below by Dr. X. Du, Cardiovascular Research Unit, Hugh Robson Building, George Square, Edinburgh, Scotland and his assistance and advice is gratefully acknowledged. Rats (Sprague Dawley strain) weighing 200 to 250g were anaesthetised with thiobutabarbital (60mg/kg, i.p.). The thorax was opened and a cannula inserted and tied into the ascending aorta for retrograde coronary perfusion (Langendorff technique). Hearts were perfused at 4.5ml/min with a modified Krebs-Henseleit solution, KHS, (Dart *et al.*,1984), gassed with oxygen and with pH adjusted to 7.4 with CO_2. Temperature at the point of entry into the aorta was 37.5°C. A polyethylene

cannula was introduced into the heart through the inferior vena cava for collection of coronary venous effluent. The left cervicothoracic ganglion and exciting cardiac nerves were dissected free for electrical stimulation. Stimulation was performed with bipolar platinum electrodes with a frequency of 5Hz for 1min. Hearts were subjected to 40min stop-flow ischaemia during which time their temperature was maintained by covering the heart with a thermostatted chamber. Coronary venous effluent was collected for 1.5min after 2x1min periods of nervous stimulation, with a 15min interval between each period, and for 3x1min periods by reperfusion following stop-flow ischaemia. Oxaprotiline (300nM) was introduced 15min prior to the second nervous stimulation and was present throughout the subsequent experiment. Hearts were weighed at the end of each experiment. Samples for noradrenaline estimation were placed on ice and stabilised by addition of an equal vol of perchloric acid to give a final concentration of 6N. All samples from these experiments were assayed by the radioenzymatic method of Da Prada and Zurcher (1976) by Miss M. Millar, Cardiovascular Research Unit, and this is gratefully acknowledged.

2.10 Fura-2 measurement of cytosolic free Ca^{2+} in suspensions of chromaffin cells.

Cells were plated at a density of $1x10^6$ cells/ml in petri dishes. After a minimum of 3 days in culture the media was removed and replaced with 5ml DMEM plus 1% (w/v) BSA at 37°C. The cells were gently scraped off the dish and incubated at room temp with 2μM Fura-2/AM, with gentle shaking. After 1h the cells were spun down and washed 2x with Ca^{2+}-free KRB at 37°C. Cells were finally resuspended at a density of $1x10^6$ cells/ml. Loaded cells were kept in the dark on ice to minimise leakage of the fluorescent indicator. All assays were performed at 37°C using a thermostatted cuvette holder fitted with a magnetic stirrer. $[Ca^{2+}]_i$ was measured as follows: small aliquots of cells, approximately $2x10^5$ were transferred to cuvettes containing KRB or Ca^{2+}-free KRB (to a final vol of 2.5ml). Cells were allowed to equilibrate for 5min with continuous stirring. Continuous monitoring of Fura-2 fluorescence was carried out in a Perkin-Elmer LS3B spectrometer linked to an IBM PC. Software supplied by Perkin-Elmer Ltd provided the means of controlling the spectrometer, driving the excitation monochromator between 2 wavelengths alternately (340 and 380nm) whilst the emission monochromator remained fixed at 510nm. Recording was paused to allow

addition of secretagogues. Following each experiment 1mM $C_{12}E_9$ was added to the cells allowing measurement of Rmax to be recorded. 50µl of 0.2M EGTA and 1M Tris (pH 10.2) was then added to reduce the Ca^{2+} concentration to subnanomolar levels, thus allowing Rmin to be recorded.

Data was collected and ratioed. Calcium concentration was determined using the equation of Grynkiewicz *et al.* (1985) and a Kd value of 225nM.

$$[Ca^{2+}]_i = \frac{R - Rmin}{Rmax - R} \times Kd \times SFB$$

SFB is the ratio of the Fura-2 fluorescence values at 380nm in Ca^{2+}-free and Ca^{2+}-saturated solutions. This was determined as described by Malgordli *et al.* (1987) using reference standards of suspensions of intact cells in which $[Ca^{2+}]_i$ was modified to either very low or very high levels.

Chapter Three

Isolation of Bovine Adrenal Chromaffin Cells

3.1 Introduction

Isolated adrenal medullary chromaffin cells have long been used to study synthesis, storage and secretion of catecholamines *in vitro* (Douglas *et al.*, 1967). Many different methods for isolation of chromaffin cells and maintenance in culture have been described (for review of methods see Livett, 1984). Two methods utilised in this study are discussed, and characterisation of a functional chromaffin cell system is described.

3.2 Results

3.2.1 Isolation.

Both isolation methods described (Chapter 2.) gave an optimum yield of up to 50 x 10^6 cells/ gland. More than 95% of these were viable, as shown by exclusion of trypan blue. This is comparable with yields obtained by other workers, for example, Fenwick *et al.* (1978) and Wilson and Viveros (1981).

The collagenase digestion step is critical in obtaining a high yield of viable cells. The time required for collagenase digestion was varied from 15 to 30min depending on the enzymic activity of the particular collagenase preparation used. Most collagenase preparations also contain a number of protease activities which are highly variable. Thus not all batches of collagenase could be relied upon to behave in the same way and numerous batches tested caused overdigestion of tissue and subsequent cell lysis. This could be prevented to some extent by altering the collagenase digestion time and keeping the concentration used to a minimum. The problems of collagenase overdigestion have been reported elsewhere (Livett, 1984; and Almazan *et al.*, 1984) and it would seem that it is the contaminants in the mixture that are responsible rather than the collagenase itself.

The second method employed consistently gave higher yields than the first method, possibly due to a more reliable collagenase preparation. It is important to include DNAse type 1 in the digestion cocktail to minimise the amount of filamentous material present and prevent cell aggregation. It should be noted that once isolated, chromaffin cells obtained from either method behaved identically under all conditions described.

3.2.2 Purification of cells.

Chromaffin cells have a different buoyant density and different sedimentation properties from the much smaller erythrocytes or lipid-containing cortical cells, enabling them to be purified on a self-generating Percoll gradient. The top 5ml of the gradient which comprises cell debris was discarded. Below this is found a

broad band of cortical cells (density 1.054g/ml), which was also discarded. These cortical cells cannot be distinguished from chromaffin cells by their appearance in the light microscope. Comparison of cortisol with catecholamine content in this fraction, however, demonstrates that this band contains the majority of cortical cells (Table 3.1). Chromaffin cells form a broad band of density about 1.06g/ml in Percoll. They were collected from a density of approximately 1.06g/ml to 1.08g/ml as determined using density gradient marker beads. Below this is a thin band of erythrocytes, density 1.13g/ml.

Purification of chromaffin cells from contaminating cortical cells and erythrocytes can be demonstrated by measurement of cortisol and catecholamines in each fraction. Table 3.1 shows that the chromaffin cell fraction contains the highest level of catecholamines compared with other fractions. Furthermore, contamination with cortical cells is minimal.

3.2.3. Cell morphology.

The morphology of purified chromaffin cells was examined after 0, 1, 2 and 3 days in culture with a phase contrast microscope. On day 0, only a few hours after isolation, chromaffin cells are beginning to attach to the culture wells and are spherical in shape. The majority of cells are phase bright with large nuclei and a granular cytoplasm, and are about 20μm in diameter; there are a few much smaller contaminating erythrocytes. After 24h in culture the cells have begun to spread out over the plastic surface and have a tendency to form into adherent chains. Within the next 2 to 3 days the cells are firmly attached and have flattened out. When seeded at low density (0.5×10^6/ml) the cells send out axon-like processes which are a characteristic feature of chromaffin cells grown in the presence of serum as shown in Fig 3.1. Serum supplementation is required for cell attachment but after this time (2 days) cells can be maintained in serum-free medium.

3.2.4. Maintenance of cells in culture.

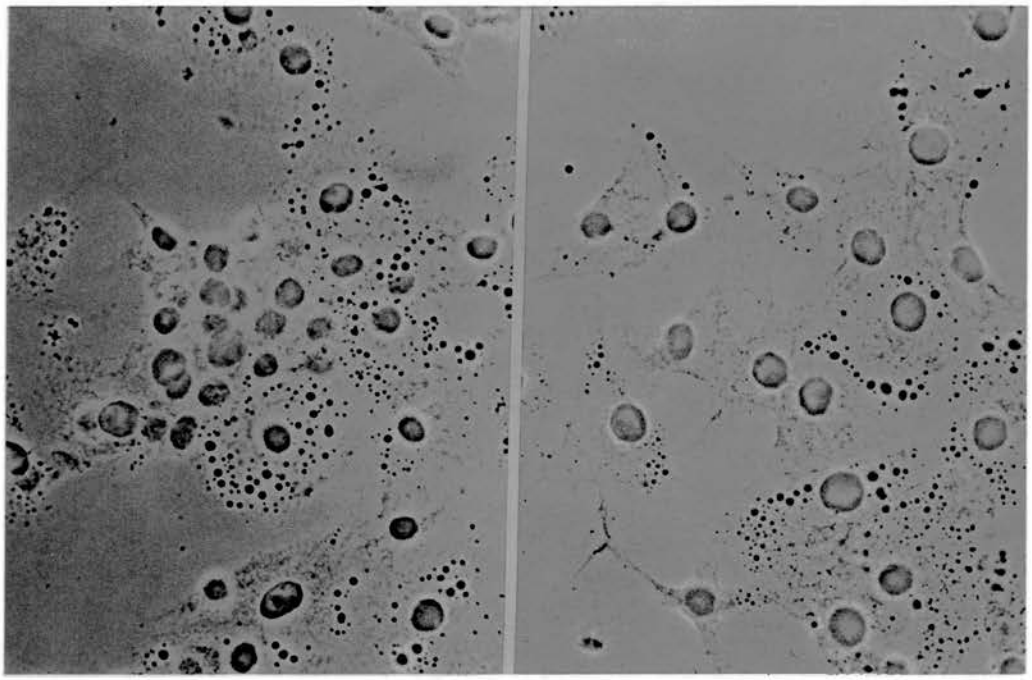
Under the conditions described chromaffin cells maintained a constant catecholamine content, as shown in Fig. 3.2, for up to 9 days in culture. Other workers report that chromaffin cells retain their catecholamine content and functional properties for at least 3 weeks (Wilson and Viveros, 1981). I found, however, that after more than 7 days in culture chromaffin cells tend to lose their ability to respond to secretagogues (Fig. 3.3) and an increasing number of cells are seen with large vacuoles in the cytoplasm, indicative of autolysis. Therefore

Table 3.1 Isolation of chromaffin cells.

	Total Yield of cells/gland $x10^6$	Cortisol nmol/10^6 cells	Total CA nmol/10^6 cells	Adrenaline nmol/10^6 cells	Noradrenaline nmol/10^6 cells	Ratio
"Debris"	37.5 ± 20	30.0 ± 4	27.6 ± 11	8.6 ± 8	19.0 ± 5	0.45
Cortical cells	77.0 ± 7	61.0 ± 18	22.4 ± 10	10.7 ± 3	11.7 ± 6.5	0.9
Chromaffin cells	51.8 ± 19	15.0 ± 0.4	125.4 ± 6.8	102.3 ± 11	23.0 ± 8	4.4
Erythrocytes	2.75 ± 1.7		11.0 ± 6	3.2 ± 6	7.8 ± 10	0.41

Table 3.1 A crude cell suspension, isolated by collagenase digestion of bovine adrenal glands, was fractionated by centrifugation on a self-generating Percoll gradient. Values are expressed as means ± SE of 5 determinations.

Fig 3.1 Phase-contrast micrograph of isolated chromaffin cells after 3 to 4 days in culture. Cells were plated at a density of 0.5×10^6 cells/well, at which density they produced axon-like processes, characteristic of chromaffin cells in culture after a few days.



chromaffin cells were never used for experiments beyond day 7.

As discussed, chromaffin cells require at least 1 day in culture to adhere firmly to the culture surface. Furthermore, freshly isolated cells show a relatively poor secretory response to nicotine, but this improves with time, reaching a peak on the third or fourth day of culture. It has been suggested (Almazan *et al.* 1984) that the impaired responsiveness after isolation results from the collagenase digestion step. This effect is not seen with K^+-evoked release, suggesting that the collagenase treatment is affecting the nicotinic receptor selectively. After a few days in culture, however, the secretory response is fully restored. In consequence, cells were always cultured for at least 2 days before experiments.

3.2.5. Biochemical studies on secretion.

The secretory response of chromaffin cells in culture was assessed by measuring the release of catecholamines in response to a variety of secretagogues. The response of the cells to the agonist nicotine was generally used as an indicator of cell viability prior to any experiments being carried out. Endogenous catecholamines were measured by spectrofluorimetry (as described in Chapter 2.). A wide variation was found in both basal and stimulated catecholamine release between different batches of cells. On average there was a 3 to 4 fold increase in catecholamine release in response to 10μM nicotine (basal release 4.1% ± 2.9 compared with a nicotine-stimulated release of 14.8% ± 4.4, n=20). In general, results given in this thesis are expressed as amount secreted as percent of total catecholamines ± SE for triplicate determinations from one batch of cells. All experiments were, however, carried out on more than one cell preparation. Incubation of chromaffin cells for 10min with a range of secretagogues resulted in a significant release of catecholamines above basal levels (Fig. 3.4). Both the physiological secretagogue, acetylcholine, and its analogue, nicotine, show a similar response. High K^+ bypasses the nicotinic receptor and elicits a response by direct depolarisation of the plasma membrane. Ba^{2+} evokes a response that is almost 3 times greater than that seen with other secretagogues. It is thought that Ba^{2+} is able to enter chromaffin cells via both voltage-sensitive and nicotinic Ca^{2+} channels and induce secretion, possibly by substituting for Ca^{2+} since Ba^{2+}-induced secretion is independent of external Ca^{2+} (Forsberg and Pollard, 1988). Alternatively, Ba^{2+} may trigger secretion by displacing Ca^{2+} from internal sites.

In most examinations of the secretory response, nicotine was used as the

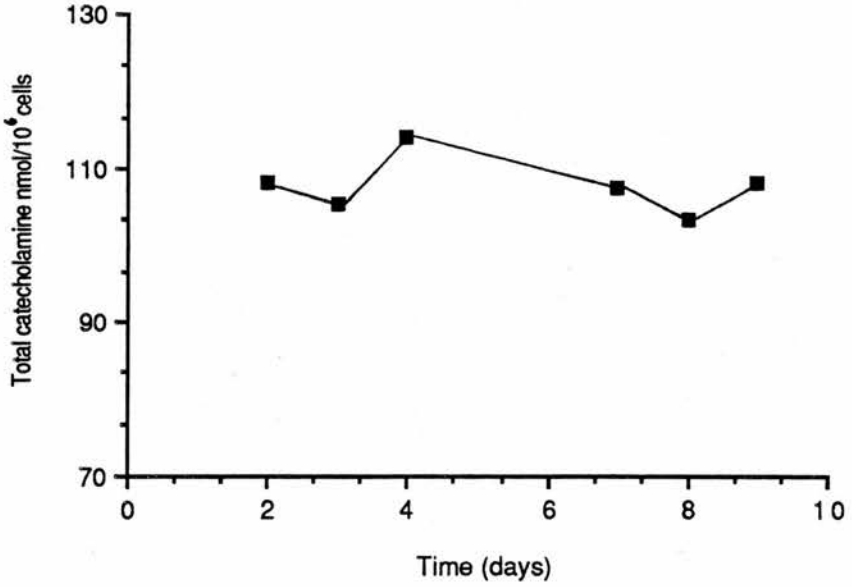


Fig 3.2 Maintenance of catecholamine content with time in culture. The total catecholamine content of chromaffin cells was measured over 9 days. Results are expressed in nmol/10^6 cells, means ± SE of triplicate determinations (error bars lie within the symbols used).

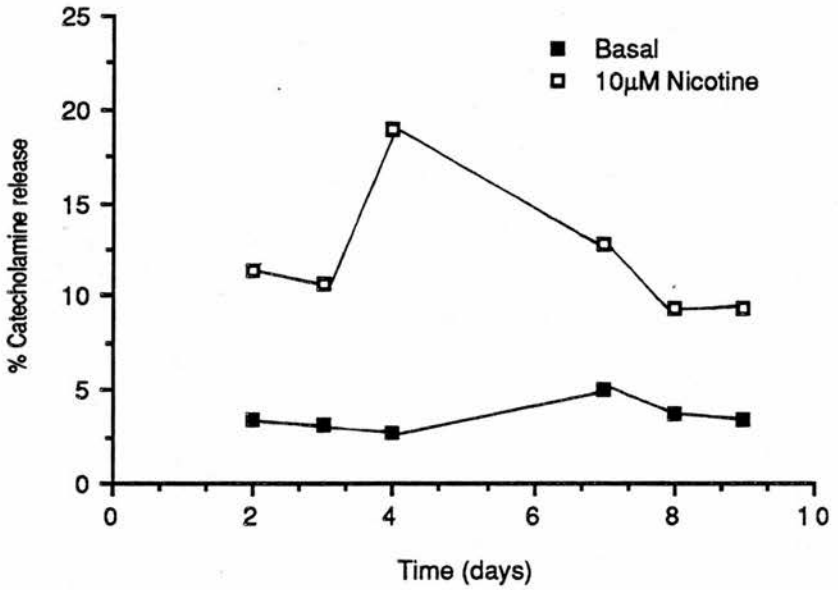


Fig 3.3 Responsiveness of cells to nicotine as a function of time in culture. Catecholamine release was measured after 10min treatment with Locke's buffer or Locke's buffer plus 10μM nicotine. The results shown are means ± SE of triplicate determinations (error bars lie within the symbols used). Basal release values have not been subtracted from release in the presence of nicotine.

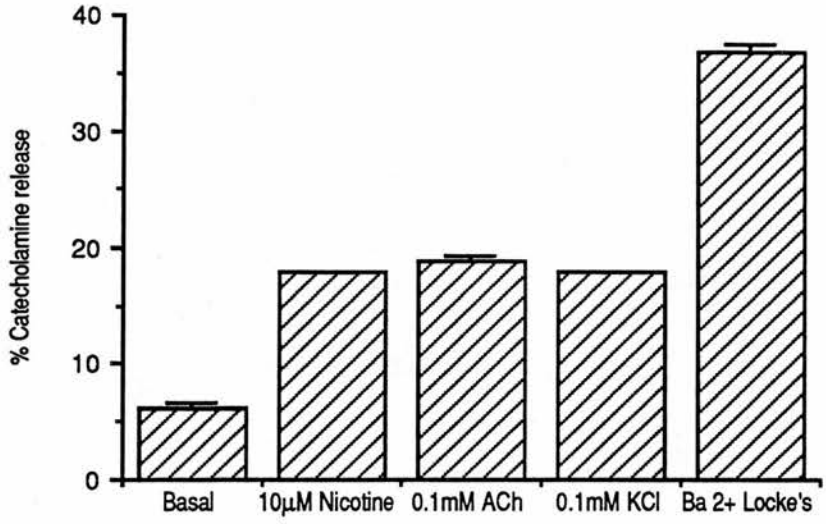


Fig 3.4 Catecholamine release in response to various secretagogues. Cells were challenged for 10min with Locke's buffer or Locke's buffer plus secretagogue. The results shown are means ± SE of triplicate determinations.

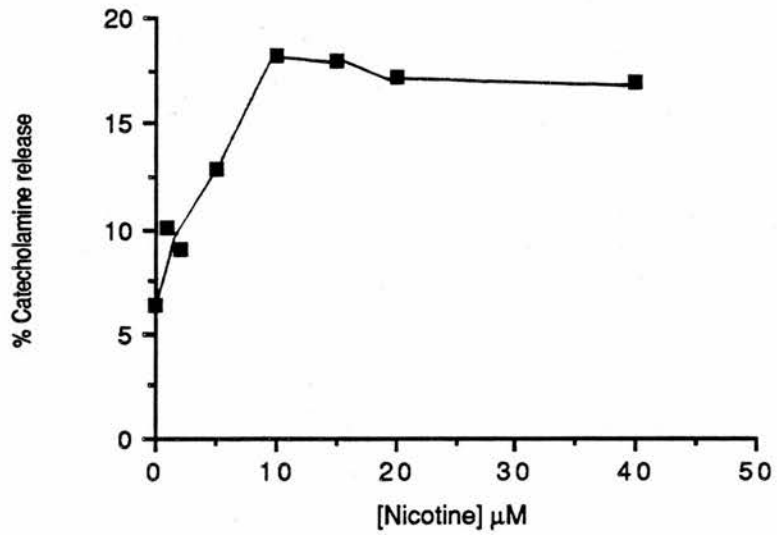


Fig 3.5 Responsiveness of cells to nicotine, 0 to 40μM. Cells were challenged for 10min with a range of nicotine concentrations. Results are expressed as percentage catecholamine release, means ± SE of triplicate determinations (error bars lie within the symbols used).

secretagogue. Fig. 3.5 shows the response to a range of nicotine concentrations, maximal secretion being evoked by 10μM nicotine after exposure for 10min. This is similar to that reported by other workers (Knight and Baker, 1983; Marley, 1988) in that the concentration of nicotine giving half-maximal release was approximately 10^{-6}M and high concentrations of nicotine released less catecholamine. The latter effect is due to desensitisation of the secretory response. Desensitisation also occurs during maintained exposure to the agonist (Fig. 3.6). Secretion is transient and returns to basal levels within a few min of exposure to nicotine. This is consistent with other studies which monitored the time course of secretion by continuous assay of ATP release (Rojas *et al.*, 1986). This type of response has been associated with a rapid nicotine-induced actin disassembly which occurs within the first min of stimulation (Cheek and Burgoyne, 1986): actin filament reassembly in the cortical region of the cell may then prevent further exocytotic release. The Ca^{2+} dependence of nicotine-evoked catecholamine secretion is shown in Fig. 3.7. When stimulated with nicotine in a Ca^{2+}-free buffer or in the presence of the Ca^{2+}-channel blocker, verapamil, secretion was completely abolished.

Chromaffin cells exist as two populations of cells, those containing mainly adrenaline, and those containing mainly noradrenaline. These two cell types respond differentially to a variety of secretagogues. In terms of absolute amount, adrenaline levels are higher than noradrenaline in the bovine adrenal medulla. For example, one cell preparation gave values of 29.2 ± 0.4 nmol/10^6 cells noradrenaline and 60.2 ± 0.7 nmol/10^6 cells adrenaline (n=9). The agonists acetylcholine, nicotine and high K^+, however, caused a preferential release of noradrenaline (Table 3.2). Ba^{2+} did not cause a preferential release of either amine.

Table 3.2

Differential release of adrenaline and noradrenaline.

	% Release	
	Noradrenaline	Adrenaline
10μM nicotine	23.5 ± 0.9	16.6 ± 0.5
0.1mM acetylcholine	22.9 ± 0.2	15.5 ± 0.1
50mM K+	22.0 ± 0.2	16.0 ±0.3
Ba2+	39.2 ± 0.6	36.6 ± 0.8

The results shown are percentage release of noradrenaline or adrenaline, measured after 10 min treatment with a range of secretagogues and expressed as means ± SE of triplicate determinations.

3.2.6. Identification of a high affinity uptake system for catecholamines.

A high affinity uptake system for noradrenaline was first characterised in chromaffin cells by Kenigsberg and Trifaró (1980). Uptake of [^{3}H] noradrenaline into chromaffin cells was followed for 24h (Fig 3.8). Uptake was found to be linear over the first 8h. The rate of uptake, however, appeared to be very slow compared to that reported by Kenigsberg and Trifaró (1980). They reported uptake of approximately 2 to 3 pmol/5min/10^6 cells when cells were incubated with 0.2μM noradrenaline. The results shown here suggested an uptake of only 0.13 pmol/5min/10^6 cells. It was postulated that this discrepancy in the uptake of noradrenaline was due to a high extracellular concentration of cold catecholamines that would compete with the label for the uptake carrier. In order to test this cells were treated as for uptake of [^{3}H] noradrenaline but the label was omitted. Samples of the incubating medium were taken over 24h and catecholamine content determined by radioenzymatic assay (Chapter 2.). A mean value of 732 ± 19 pmol catecholamine/10^6 cells (165 ± 4.5 pmol noradrenaline and 567 ± 13 pmol adrenaline, n=15) or 1.2 μM in the medium, was obtained from three cell preparations. This value remained relatively constant for the whole time course. When the concentration of cold catecholamines is taken into consideration the rate of uptake is similar to that previously reported by Kenigsberg and Trifaró (1980), a value of 3.6 ± 0.2 pmol noradrenaline/5min/10^6 cells (n=6) being calculated from my data. It was necessary, however, to use prolonged incubations in order to allow cells to accumulate sufficient radioactivity for subsequent experiments.

The uptake process demonstrated all the reported features of the high affinity

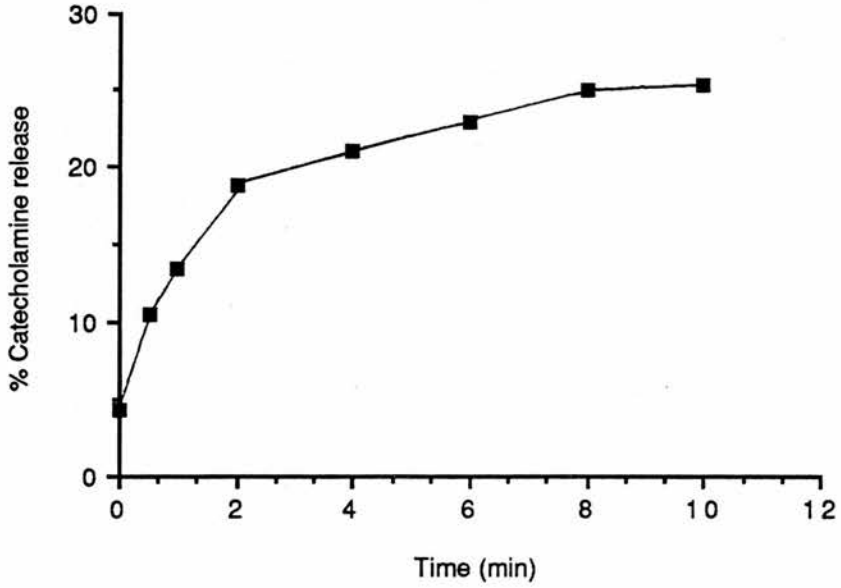


Fig 3.6 Percentage catecholamine release in response to 10μM nicotine with time. Results shown are means ± SE of triplicate determinations (error bars lie within the symbols used).

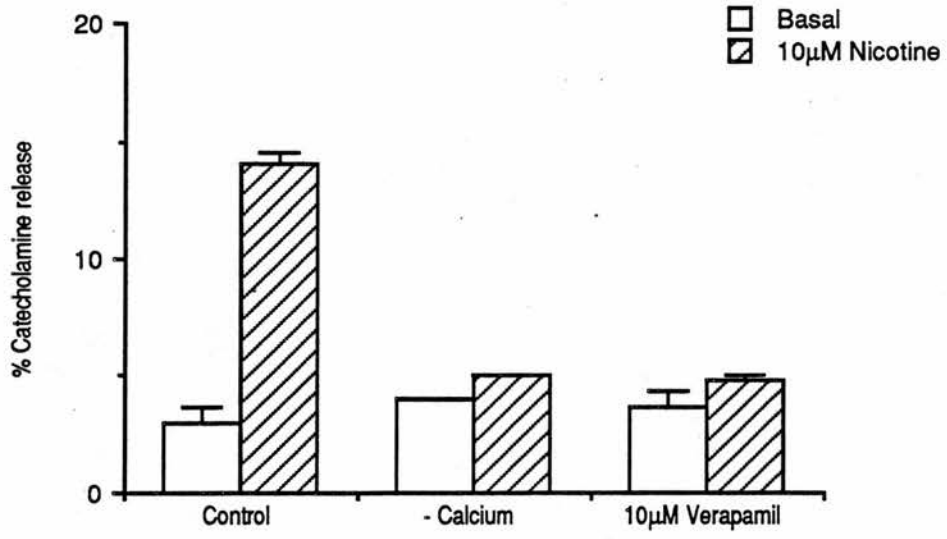


Fig 3.7 Calcium dependence of nicotine-stimulated exocytosis. Catecholamine release was measured after 10min treatment with Locke's buffer or Locke's buffer plus 10μM nicotine in the presence or absence of extracellular calcium or plus 10μM verapamil. The results shown are means ± SE of triplicate determinations.

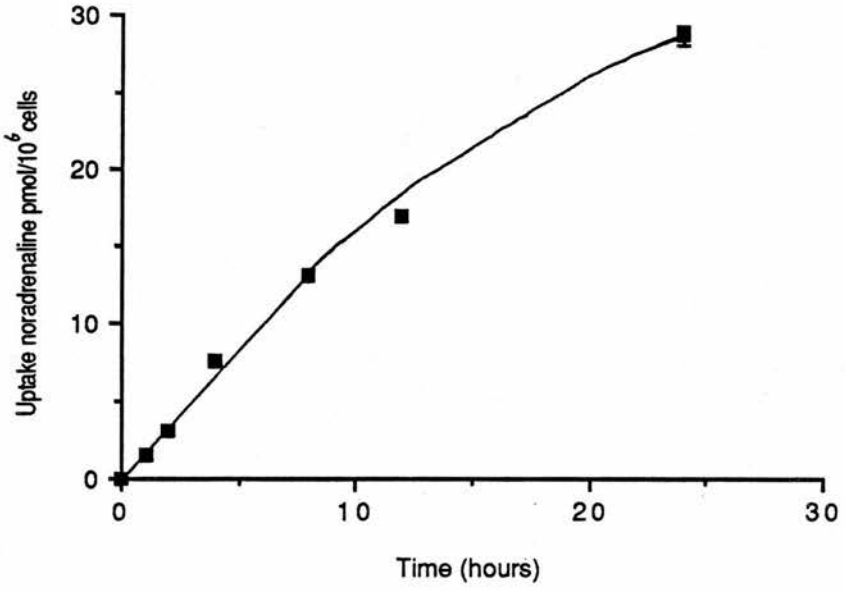


Fig 3.8 Uptake of [^{3}H] noradrenaline. Cells were incubated with [^{3}H] noradrenaline for up to 24h. Uptake is expressed as pmol [^{3}H] noradrenaline/10^6 cells when culture media were replaced with DMEM plus 0.2μM [^{3}H] noradrenaline (Chapter 2). Values shown are means ± SE of 6 determinations.

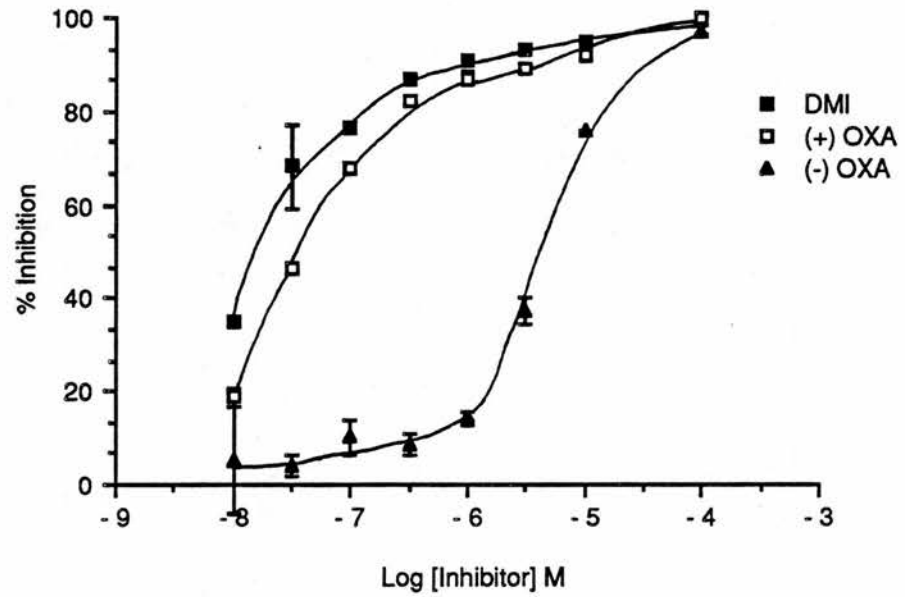


Fig 3.9 Inhibition of uptake of [^{3}H] noradrenaline by uptake1 blockers. Chromaffin cells were incubated with [^{3}H] noradrenaline for 12h in the presence of different concentrations of desipramine (DMI) and, (+) and (-) oxaprotiline (OXA). Results are expressed as percent inhibition, uptake in the absence of inhibitor being 100%. Results shown are means ± SE of 6 determinations.

uptake system described by Kenigsberg and Trifaró (1980). Uptake was blocked by low temperature, metabolic inhibition and sodium depletion (results not shown). The $uptake_1$ inhibitors desipramine and, (+) and (-) oxaprotiline blocked the uptake of [^{3}H] noradrenaline in chromaffin cells with a similar sensitivity to that reported previously (Banerjee *et al.*, 1987) and also reported for isolated rat synaptosomes (Waldmeier *et al.*, 1982). Fig. 3.9 shows the sensitivity of uptake to these drugs over a concentration range of 10^{-8} to 10^{-3}M. Oxaprotiline exists as two isomers, the (+) enantiomer being up to 1000 times more potent than the (-) form (Waldmeier *et al.*, 1982). In chromaffin cells (Fig 3.9) there is a 200 fold difference in the sensitivity of the two isomers for the uptake carrier. Both forms are, however, identical in their non-specific effects on adrenergic responses. Therefore, this sensitivity of (+) oxaprotiline over (-) oxaprotiline to block uptake of [^{3}H] noradrenaline must be due to its affinity for the uptake carrier rather than to any non-specific action of the drug. Both (+) oxaprotiline and desipramine show a similar affinity for the uptake carrier, approximately 10^{-7}M being sufficient to cause 50% inhibition of uptake. Kenigsberg and Trifaró (1980) reported 60 to 80% inhibition of uptake with 10^{-7}M desipramine. In this study it was not possible to make a direct comparison with their results as only one concentration of noradrenaline was used. This concentration of desipramine is, however, low enough to be almost totally selective for the uptake carrier.

3.3. Discussion.

The evidence presented here confirms previous reports on the utilisation of isolated chromaffin cells for studies on secretory processes. Morphological studies show that the cells isolated by the methods described are a relatively homogeneous population and possess all the characteristics indicative of chromaffin cells. Catecholamines are secreted in response to various secretagogues. Acetylcholine, nicotine and high K^+ evoke secretion that is coupled to voltage-sensitive Ca^{2+} channels. Ba^{2+} elicits a much higher response that is also maintained at these high levels for a much longer time, 55% of total catecholamine having been lost after 60min (Hunter and Phillips, 1989). In contrast, the response to physiological secretagogues such as acetylcholine is transient and is essentially over within 10min. The response to the acetylcholine analog, nicotine, shows many of the characteristics of exocytosis in that release is Ca^{2+}-dependent. It is also

temperature and energy dependent (results not shown), release being abolished at low temperature or after depletion of ATP with metabolic inhibitors.

These studies have also identified an uptake system for noradrenaline that is similar to that previously described in chromaffin cells (Kenigsberg and Trifaró, 1980) and in PC12 cells (Bönisch *et al.*, 1984) Uptake of [^{3}H] noradrenaline was energy and Na^+-dependent and sensitive to blockade by the tricyclic antidepressants desipramine and oxaprotiline. The existence of oxaprotiline as two isomers with differing sensitivities for the $uptake_1$ carrier has proved particularly useful in distinguishing the uptake-blocking capacity of the drug from other non-specific effects.

In conclusion, the results presented here confirmed that chromaffin cells can be maintained in culture for at least one week while retaining physiological properties. Furthermore, these results confirm predictions that isolated chromaffin cells will be a suitable model for studying the effects of ischaemia on an isolated cell system.

Chapter Four

A Model of Anoxia in Isolated Chromaffin Cells

4.1 Introduction.

Having established a viable population of cells that demonstrated all the morphological and functional properties of chromaffin cells, it was possible to investigate the conditions that would mimic the ischaemia-induced overflow of noradrenaline observed in isolated rat hearts. Ischaemia itself could not be imposed since this, by definition, implies a cessation or reduction of blood flow. Hypoxia, where the pO_2 in the ischaemic zone is less than 20mmHg, is a major feature of myocardial ischaemia and could be produced in isolated cells. Anoxia, itself, where the pO_2 equals 0mmHg, is only found in total global ischaemia.

Schömig *et al.* (1987) have demonstrated that the release of noradrenaline evoked by anoxia in rat hearts is essentially the same in mechanism of release as that observed during myocardial ischaemia, imposed by a cessation of blood flow. The conditions used to induce anoxia were perfusion of the hearts with oxygen-free and glucose-free media, or perfusion with glucose-free medium supplemented with 1mM cyanide. A similar method of cyanide intoxication was used by Kauppinen and Nicholls (1986) to induce anoxia in isolated cortical synaptosomes.

Non-neurogenic release of adrenal catecholamines in neonatal rats has also been studied using conditions of hypoxia, that is, 7% oxygen for 2h (Slotkin and Seidler, 1988), and Bülbring (1948) used conditions of nitrogen-saturation or cyanide injection to evoke release in isolated perfused adrenal glands. This latter model has considerable relevance to a possible model of anoxia in chromaffin cells. It was proposed, therefore, to simulate ischaemia in chromaffin cells either by using conditions of substrate-free anoxia, or by chemical metabolic inhibition. Non-exocytotic release could be characterised by its calcium-independence and sensitivity to $uptake_1$ blockade (see Chapter 1.).

4.2 Results.

4.2.1 Catecholamine release under conditions of 'anoxia' or metabolic inhibition.

Three different experimental designs were used to study the effects of anoxia on catecholamine release from chromaffin cells.

i) Hypoxia (pO_2<20mmHg) was induced using a nitrogen-flushed chamber and a cell medium of glucose-free Locke's buffer which had been deoxygenated and then regassed with nitrogen.

ii) Total anoxia i.e pO_2 of 0mmHg could not be achieved by deoxygenation. Anoxia

could, however, be mimicked using metabolic inhibition, induced by using 1mM sodium cyanide and 1mM iodoacetate in Locke's buffer in a normal atmosphere.
iii) An alternative model of metabolic inhibition was investigated using 1μM antimycin A and 6mM 2-deoxy D-glucose to distinguish between a real release of catecholamines in response to anoxia and any non-specific effects induced by the inhibitors themselves.
Initial experiments monitored catecholamine release for up to 2h since, in isolated perfused hearts, noradrenaline overflow did not become significant until at least 15min after the onset of ischaemia (Dart *et al.*, 1986). Fig 4.1 shows the release of catecholamines in response to anoxia or metabolic inhibition. A significant release ($p<0.001$) of catecholamines was observed within the first 10min of anoxia or metabolic inhibition. This release was 2 to 3 times higher than basal levels and the catecholamine was not reaccumulated over at least 2h. Beyond this time non-specific cell lysis occurs and cells tend to lift off the culture plates, as observed by phase contrast microscopy. Over the first hour of anoxia, however, the chromaffin cells remained intact and morphologically identical to control cells, demonstrated by their ability to exclude trypan blue. A similar release of catecholamines was observed under all three conditions suggesting this is a real response and not due to some non-specific drug action. To discount the possibility that this observed release was limited to one cell preparation this time course was repeated on several different batches of cells. Fig 4.2 shows the responses obtained from three different cell preparations under conditions of metabolic inhibition, with 1mM cyanide and 1mM iodoacetate. All three models gave similar, and reproducible, responses in different cell preparations.
All subsequent experiments were carried out using metabolic inhibition (1mM cyanide and 1mM iodoacetate) to simulate and study anoxic catecholamine release. In order to prevent metabolism of catecholamines occurring under these conditions, which are aerobic compared to the anoxic model, cells were pre-incubated with 0.1mM pargyline for 15min prior to metabolic inhibition. Pargyline was used to inhibit MAO activity as previously described by Bönisch *et al.* (1984). There was very little difference ($p>0.1$) in catecholamine release in the presence of metabolic inhibitors with or without pargyline (Fig 4.3) indicating that metabolism of catecholamines under these conditions is not a problem. It is also further evidence that the cells retain their integrity since non-specific cell lysis would result in the release of MAO into the extracellular

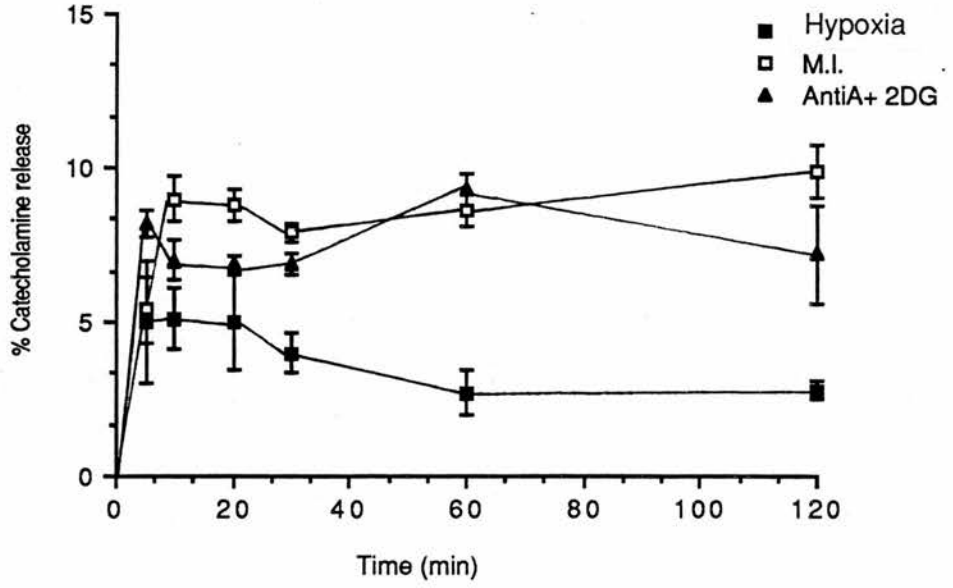


Fig 4.1 Time course of catecholamine release under conditions of hypoxia or metabolic inhibition. Cells were treated for 2h with N_2-saturated, glucose-free Locke's buffer (hypoxia) or Locke's buffer plus metabolic inhibitors (1mM sodium cyanide and 1mM iodoacetate, M.I., or 1μM antimycin A and 6mM 2-deoxy D-glucose, AntiA + 2DG). Results are expressed as percentage catecholamine release above basal levels, means ± SE of triplicate determinations.

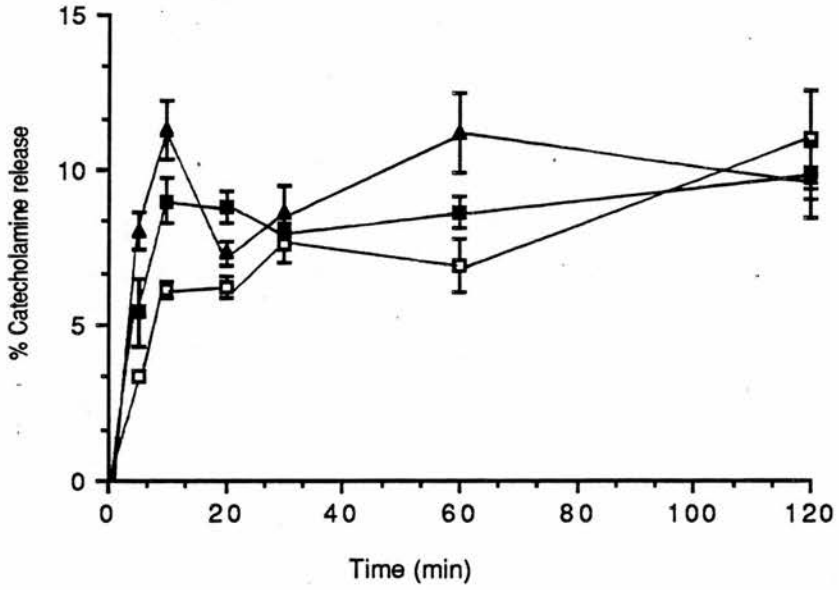


Fig 4.2 Response of three different cell preparations to metabolic inhibition (1mM sodium cyanide and 1mM iodoacetate). Results are expressed as percentage catecholamine release above basal levels, means ± SE of triplicate determinations.

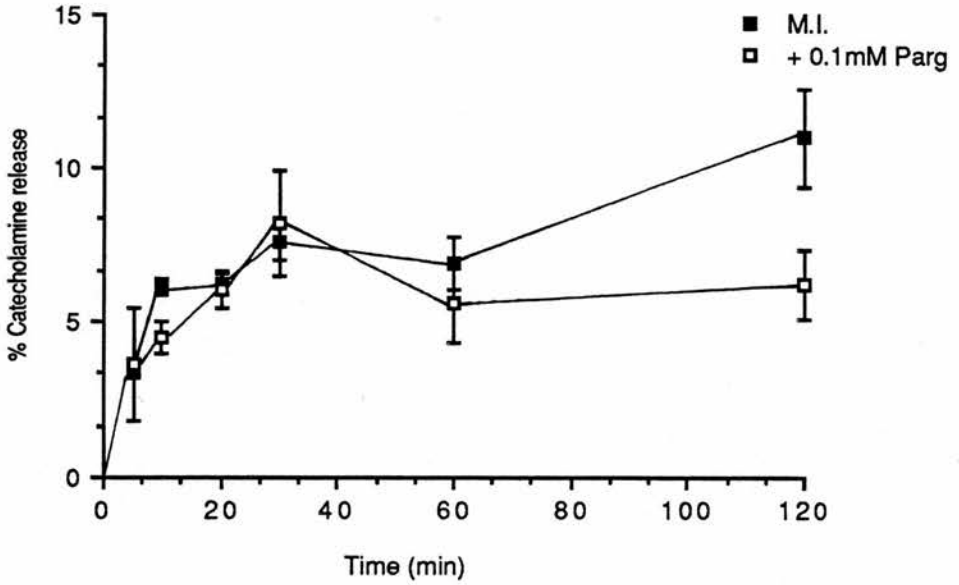


Fig 4.3 Release of catecholamines in response to metabolic inhibition in the presence or absence of the MAO inhibitor, pargyline. Cells were preincubated for 15min with 0.1mM pargyline prior to the addition of Locke's buffer plus metabolic inhibitors. Results are expressed as percentage catecholamine release above basal levels, means ± SE of triplicate determinations.

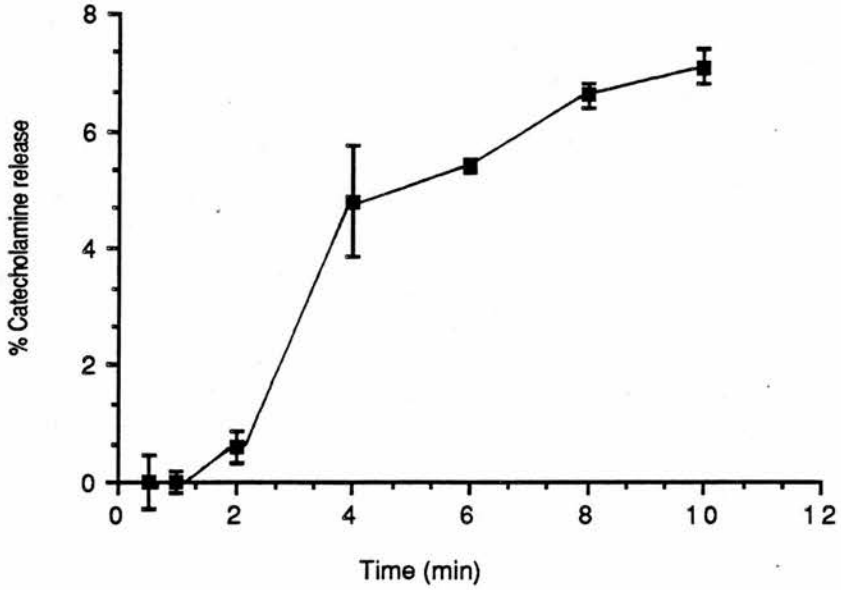


Fig 4.4 Ten minute time course of catecholamine release under conditions of metabolic inhibition. Results are expressed as percentage catecholamine release above basal levels, means ± SE of triplicate determinations.

medium with subsequent metabolism of any released catecholamines. As with the response to nicotine, catecholamine release under conditions of metabolic inhibition reached a maximum within 10min (Fig 4.4). Unlike the nicotinic response (Fig 3.6), however, where catecholamine release is essentially over within the first 60sec, there is a time lag of 2min before any significant ($p<0.001$) catecholamine release is observed (Fig 4.4).

4.2.2 Characterisation of catecholamine release.

As discussed, carrier-mediated efflux of noradrenaline from ischaemic rat hearts was characterised by its calcium-independence and sensitivity to the uptake$_1$ inhibitor, desipramine (Dart *et al.*, 1987). Once a model of "ischaemia-induced" catecholamine release had been established in chromaffin cells these same criteria were applied. Chromaffin cells were treated with metabolic inhibitors (1mM cyanide and 1mM iodoacetate) in Locke's buffer in the presence or absence of extracellular calcium or in the presence of the calcium-channel blocker, verapamil (Fig 4.5). Catecholamine release induced by metabolic inhibitors was independent of calcium and unaffected by the calcium channel blocker, verapamil, unlike nicotine-induced catecholamine release (Fig 3.7).

The second feature of carrier-mediated efflux, its sensitivity to uptake blockade, was also examined. Cells were pretreated with 100nM desipramine for 15min prior to addition of Locke's buffer plus metabolic inhibitors. Desipramine was present throughout the experiment. As shown (Fig 4.6) there was no significant reduction in release in the presence of 100nM desipramine ($p>0.01$); if anything, there was slight enhancement of release, perhaps due to inhibition of re-uptake. This lack of an inhibitory effect by desipramine raised the question of whether this concentration of drug was really sufficient to block the cellular uptake mechanism under these conditions, particularly as the extracellular catecholamine concentration is higher (approximately 10μM) than it was during the experiments on blockade of uptake of [^{3}H] noradrenaline (approximately 1μM), described in Chapter 3. 100nM desipramine was found to be sufficient to inhibit uptake of [^{3}H] noradrenaline by at least 60% by Kenigsberg and Trifaró (1980), and see Fig 3.9. The effect of raising the extracellular non-radioactive catecholamine concentration on the ability of uptake$_1$ blockers to inhibit uptake of [^{3}H] noradrenaline was, therefore, examined. Uptake of [^{3}H] noradrenaline in the presence or absence of desipramine, (+) and (-) oxaprotiline was measured as

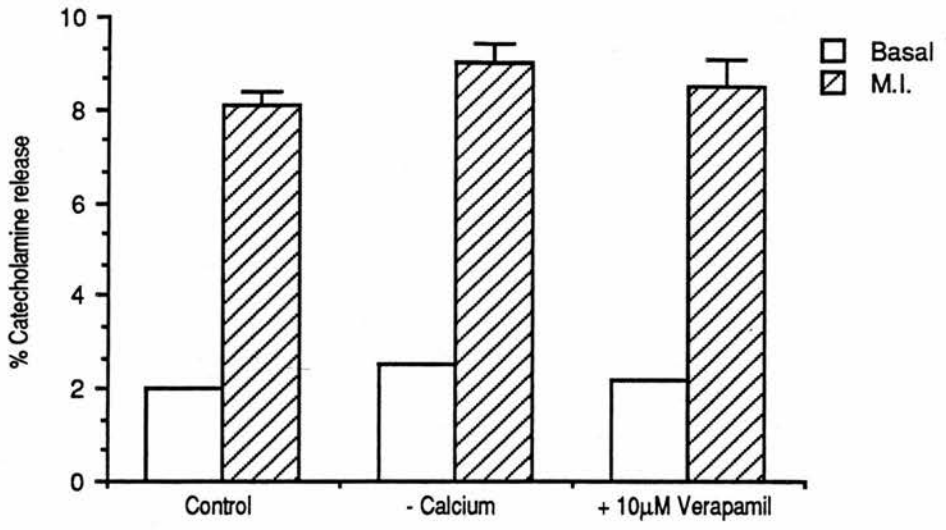


Fig 4.5 Calcium independence of catecholamine release in response to metabolic inhibition. Results are expressed as percentage catecholamine release after 10min treatment with Locke's buffer or Locke's buffer plus metabolic inhibitors in the presence or absence of calcium or 10μM verapamil. Results are means ± SE of triplicate determinations.

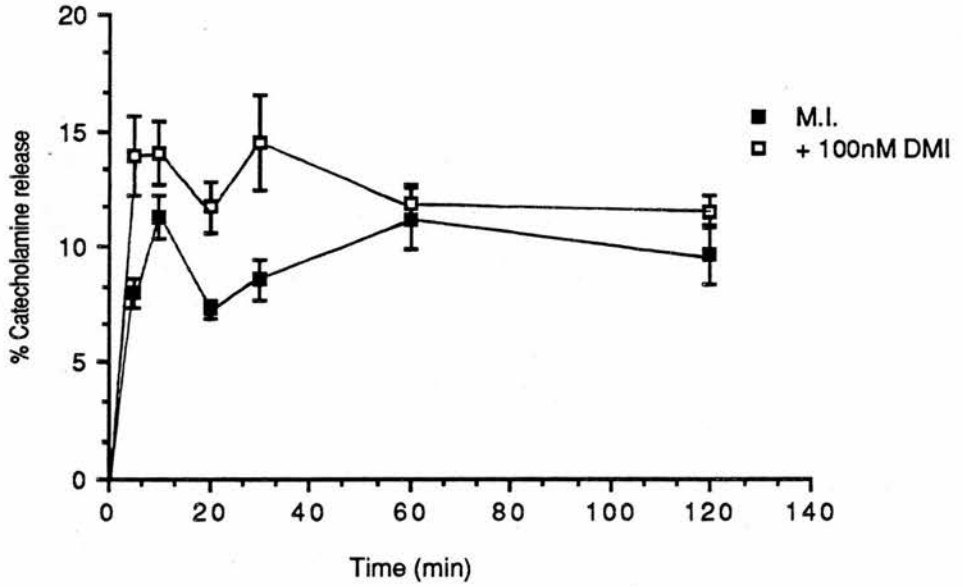


Fig 4.6 Effect of 100nM desipramine on catecholamine release in response to metabolic inhibition. Cells were pretreated for 15min with 100nM desipramine prior to the addition of Locke's buffer plus metabolic inhibitors. Results are expressed as percentage catecholamine release above basal levels, means ± SE of triplicate determinations.

described in Chapter 2. Non-radioactive noradrenaline and adrenaline were included in the incubating medium at a concentration and ratio within the range released by either nicotine-stimulation or metabolic inhibition (3.5μM noradrenaline and 6μM adrenaline). These added catecholamines had no significant effect ($p>0.1$) on the ability of the uptake$_1$ blockers to inhibit uptake of [^{3}H] noradrenaline (Fig 4.7).

Desipramine is a competitive inhibitor of the carrier, exerting its inhibitory effect by direct competition with an amine substrate for its binding site (Bönisch, 1984). Although non-radioactive catecholamines in the medium did not appear to have any effect on inhibition of uptake, it is possible that intracellular catecholamines released during metabolic inhibition could compete with desipramine for the uptake carrier and reduce its effectiveness as an inhibitor. Therefore, the effect of a range of desipramine concentrations on the release of catecholamines under conditions of metabolic inhibition was examined. 1μM desipramine was sufficient to inhibit catecholamine release by 50% (Fig 4.8). However, it was found that desipramine inhibited nicotine-evoked catecholamine release to the same degree. This latter effect has previously been reported in the perfused adrenal gland (Wakade and Wakade, 1984), catecholamine secretion being reduced in a dose-dependent manner. This ability of desipramine to inhibit secretion of catecholamines has also been reported in the isolated perfused guinea pig heart (Richardt *et al.*, 1988), where nicotine-induced noradrenaline release was almost completely abolished by 1μM desipramine. Nisoxetine, another uptake$_1$ blocker had a similar effect. In the case of chromaffin cells, desipramine cannot be used to distinguish between nicotine-stimulated exocytosis and catecholamine release induced by metabolic inhibitors. If one assumes that it will be an effective blocker of carrier-mediated efflux at the same low concentrations as are effective in inhibiting carrier-mediated uptake (Fig 4.7), these observations suggest that the carrier is not involved. Furthermore, the observations raise questions about noradrenaline overflow blockade in ischaemic rat hearts, since it is hard to show whether inhibition of overflow by desipramine is due to its ability to block uptake$_1$, or might be due to some other non-specific action of the drug.

To resolve these questions oxaprotiline was utilised to distinguish between inhibition of the uptake carrier and other non-specific inhibition , by using the

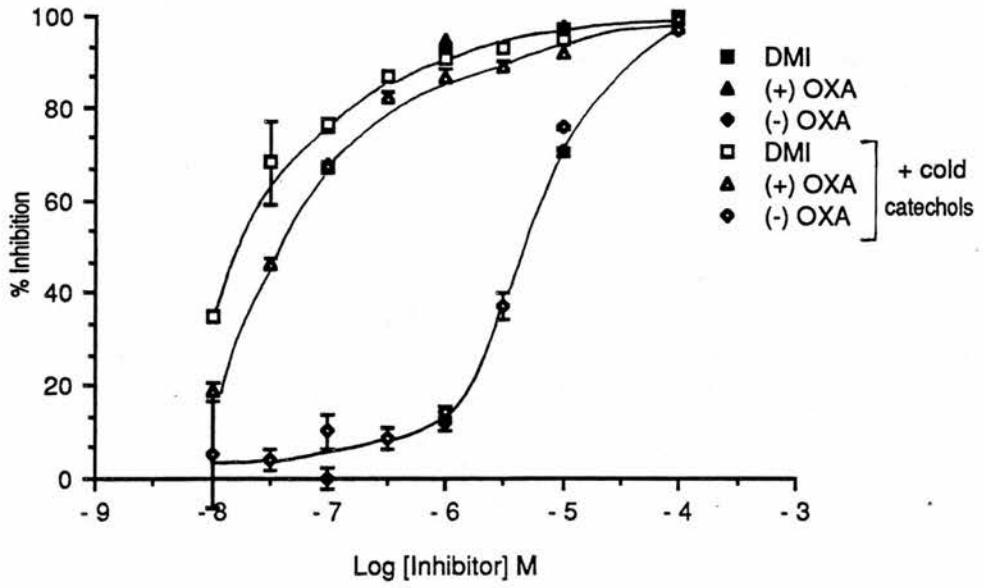


Fig 4.7 Effect of extracellular catecholamines on blockade of uptake of [^{3}H] noradrenaline. Cells were incubated with [^{3}H] noradrenaline for 12h in the presence of different concentrations of desipramine (DMI) and, (+) and (-) oxaprotiline (OXA). Uptake was measured in the presence (+ cold catecholamines) or absence of nonradioactive extracellular catecholamines. Results are expressed as percentage inhibition, means ± SE of 6 determinations.

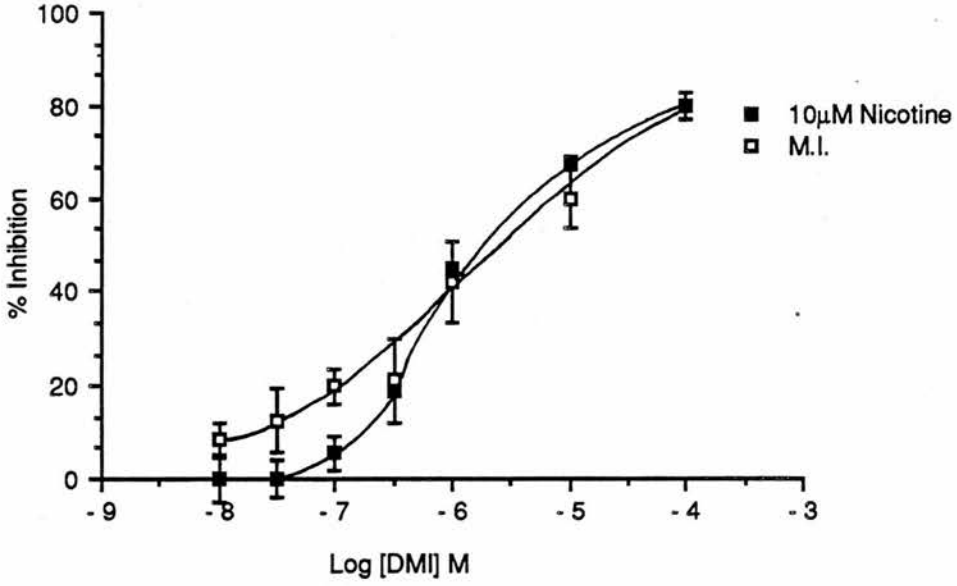


Fig 4.8 Ability of desipramine to inhibit catecholamine release in response to 10μM nicotine or metabolic inhibition. Cells were pretreated for 15min with a range of desipramine concentrations prior to addition of Locke's buffer plus 10μM nicotine or metabolic inhibitors. Desipramine was present throughout the experiment. Catecholamine release was measured after 10min and results are expressed as percentage inhibition, means ± SE of triplicate determinations.

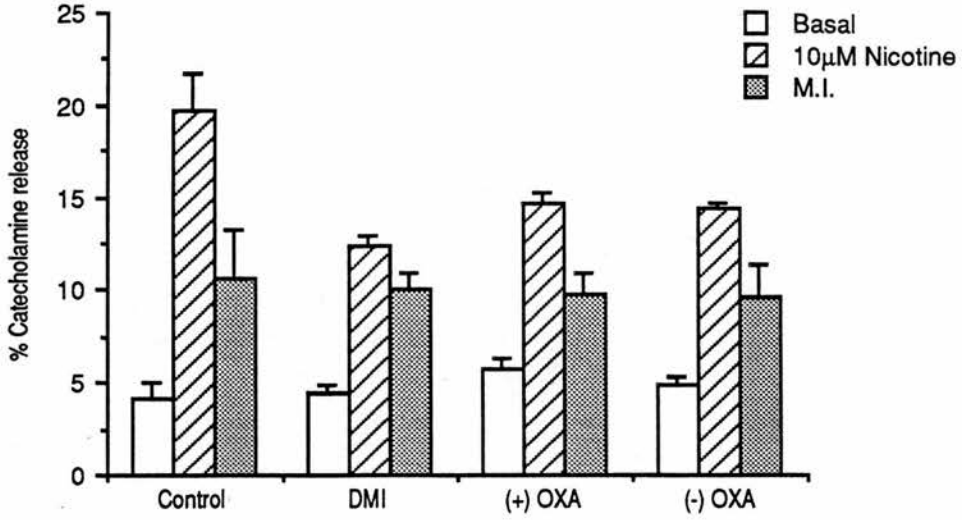


Fig 4.9 Catecholamine release evoked by 10μM nicotine or metabolic inhibitors in the presence of uptake blockers. Cells were preincubated for 15min with 100nM desipramine (DMI) or 1μM (+) or (-) oxaprotiline (OXA), prior to the addition of Locke's buffer plus 10μM nicotine or metabolic inhibitors. Drugs were present throughout the experiment. Catecholamine release was measured after 10min and results are expressed as percentage release, means ± SE of triplicate determinations.

two isomers of the drug. Chromaffin cells were preincubated for 15min with 100nM desipramine or 1μM (+) or (-) oxaprotiline (the concentration at which there was the greatest difference in the uptake blocking capacity of the two isomers, Fig 4.7). Fig 4.9 shows the catecholamine release after 10min treatment with Locke's buffer or Locke's buffer plus 10μM nicotine or metabolic inhibitors. Catecholamine release evoked by 10μM nicotine was reduced by desipramine and oxaprotiline (approximately 40% inhibition). There was no difference between release in the presence of (+) or (-) oxaprotiline suggesting that this reduced release is due to some non-specific action of the drug and not due to blockade of the uptake carrier. Catecholamine release induced by metabolic inhibition was not inhibited by desipramine, (+) or (-) oxaprotiline. The finding that 1μM desipramine also inhibited nicotine-evoked noradrenaline release in isolated guinea pig hearts (Richardt *et al.*, 1988) suggests that it may be unsafe to conclude that inhibition by this drug is an indication of a carrier-mediated efflux mechanism for ischaemia-induced noradrenaline release.

4.2.3 Sensitivity of noradrenaline overflow in perfused rat hearts to (+) and (-) oxaprotiline.

Ischaemia-induced noradrenaline overflow in rat hearts was therefore reexamined using an *in situ* model of stop-flow ischaemia. The left cervicothoracic ganglion was electrically stimulated (for 1min intervals at 5Hz) in the presence or absence of (+) and (-) oxaprotiline prior to the onset of stop-flow ischaemia in order to compare the action of the two isomers on both exocytotic and non-exocytotic noradrenaline release. Fig 4.10 shows the noradrenaline overflow in response to 3min electrical stimulation of the left cervicothoracic ganglion in the presence or absence of (+) or (-) oxaprotiline. There was no significant difference ($p>0.5$) between release in the presence of either drug compared with that observed on stimulating the ganglion in its absence. Following electrical stimulation noradrenaline efflux was allowed to return to basal levels before stop-flow ischaemia was induced. Noradrenaline overflow was then measured during the initial 3min reperfusion following 40min stop-flow ischaemia, (Fig 4.11). Ischaemia-induced noradrenaline overflow was significantly reduced in the presence of 3×10^{-6}M (+) oxaprotiline from 117 ± 21.5 pmol/g ($n=8$) to 26 ± 6.0 pmol/g ($n=6$), $p<0.01$. While there was an apparent reduction of noradrenaline overflow in the presence of (-) oxaprotiline, 74 ± 17.7 pmol/g ($n=6$), this was not significantly different to overflow in the absence of drug; $p>0.5$. This appears

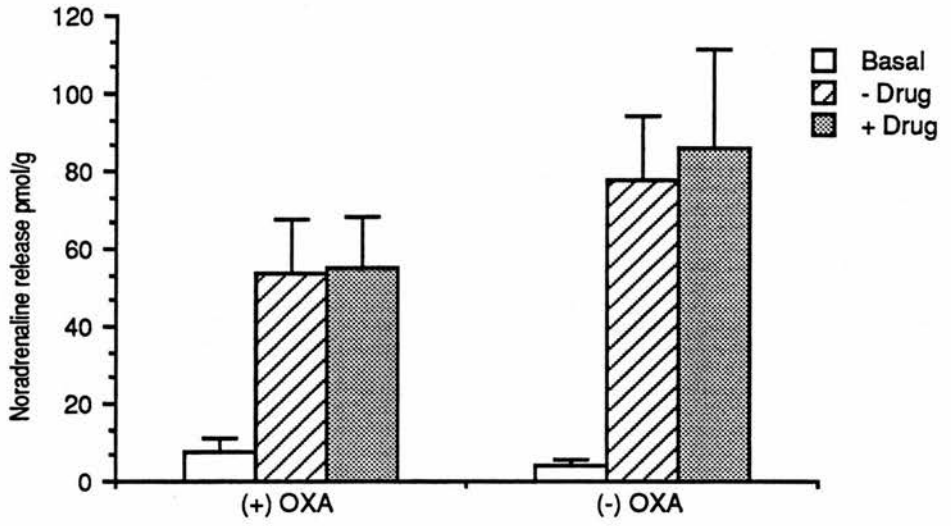


Fig 4.10 Release of noradrenaline from the perfused rat heart before (Basal) or during 3min continuous electrical stimulation (5Hz/min) of the left cervico-thoracic ganglion in the presence or absence of (+) or (-) oxaprotiline at a flow rate of 4.5ml/min. The drug was added at $3x10^{-7}$M 15min prior to the second stimulation. Cumulative release is expressed in pmol/g heart weight, means ± SE from 8 hearts.

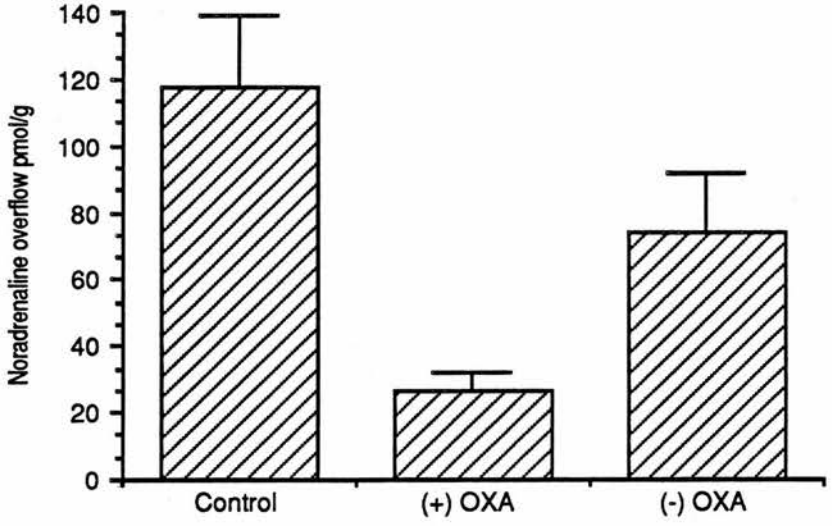


Fig 4.11 Overflow of noradrenaline from the perfused rat heart following 40min stop-flow ischaemia. Drug, 3×10^{-7}M (+) or (-) oxaprotiline was present throughout the experiment. Cumulative overflow is expressed in pmol/g, means ± SE from 6 to 8 hearts.

to confirm that the ability of (+) oxaprotiline to inhibit ischaemia-induced noradrenaline overflow in the perfused rat heart can only be attributed to its uptake$_1$ blocking activity. This provides further evidence that noradrenaline overflow in the ischaemic rat heart occurs by carrier-mediated efflux.

4.3 Discussion.

Conditions of anoxia or metabolic inhibition produced a significant release ($p<0.001$) of catecholamines above basal release in chromaffin cells (Fig 4.1). This reached a maximum of 5 to 10% of cell content above basal release within 10min, a similar magnitude to release evoked by nicotine or high K^+. Anoxia or cyanide intoxication of the isolated perfused rat heart produced an overflow of noradrenaline only after a lag period of 5 to 10min that reached a peak after 30 to 40min and then declined (Schömig *et al.*, 1987). This lag period is thought to be due to ongoing glycolysis, a significant overflow of noradrenaline only occurring after cessation of glycolytic ATP production. Reduction of cardiac glycogen stores by glucose-free preperfusion reduced this lag period. Chromaffin cells do not have significant glycogen stores so energy depletion would be expected to be more rapid. The response of perfused adrenal glands to stimulation with acetylcholine was completely abolished following 10min perfusion with cyanide and iodoacetate (Kirchner and Smith, 1969). This indicates a complete rundown of metabolic energy within this time. Furthermore, Corcoran *et al.* (1986) reported a rapid decline in the cytosolic pool of ATP in chromaffin cells with a $t_{1/2}$ of 5min, using a combination of cyanide, 2-deoxy D-glucose and β glucono 1,5 lactone. Catecholamine release in response to metabolic inhibition occurred after an initial lag phase of 2min (Fig 4.4). This may be due to an initial depletion of energy stores prior to catecholamine overflow and there may be other prerequisite metabolic changes.

Anoxia or cyanide intoxication in the perfused rat heart produced a maximum overflow of 80 pmol/g/min noradrenaline (Schömig *et al.*, 1987) which is much greater than that achieved with stimulation of the left cervicothoracic ganglion. These conditions in isolated chromaffin cells did not produce such a large increase in release, the maximal catecholamine release in response to metabolic inhibition being no greater than that evoked by nicotine.

Noradrenaline overflow in isolated rat hearts induced by anoxia is calcium-independent (Schömig *et al.*, 1987). When similar conditions were

imposed on chromaffin cells, catecholamine release was also found to be calcium-independent. Furthermore, catecholamine release was unaffected by 10μM verapamil which completely abolished nicotine-stimulated release. Noradrenaline overflow induced by cyanide intoxication in the perfused rat heart was more than 40% higher in the absence of calcium (Schömig *et al.*, 1987). The time lag was also reduced in the absence of calcium. Calcium ions have been shown to inhibit sodium entry through sodium channels so the absence of calcium would be expected to facilitate sodium entry via this route. This would lead to a more rapid rise in intracellular sodium, facilitating sodium-linked carrier-mediated efflux. Such a sequence of events, however, has yet to be demonstrated experimentally. There appeared to be a slight enhancement of catecholamine overflow from chromaffin cells in the absence of calcium (9.0 ± 0.4%, calcium-free compared with 8.1 ± 0.3%, plus calcium), but this difference is not significant ($p>1.0$). Desipramine inhibited catecholamine release induced by metabolic inhibitors but only at concentrations of 10^{-6}M and higher (Fig 4.8). Nicotine-stimulated exocytosis was also inhibited at this concentration to a similar degree. This effect has previously been reported in the perfused adrenal gland (Wakade and Wakade, 1984), secretion evoked by acetylcholine, splanchnic nerve stimulation and high K^+ being affected similarly, suggesting that exocytosis is inhibited at a common site under these conditions. In each case, secretion was inhibited by 50 to 70% by 3μM desipramine. A similar degree of inhibition is seen in isolated chromaffin cells (Fig 4.8). This inhibitory effect of desipramine has been attributed to its ability to bind to calmodulin. This was not measured directly in the perfused adrenal gland and it is possible that desipramine may have other sites of action. Desipramine has also been reported to inhibit nicotine-evoked noradrenaline release in the perfused guinea pig heart (Richardt *et al.*, 1988). However, unlike that observed in the adrenal gland, desipramine did not inhibit noradrenaline release in response to electrical stimulation (Haass *et al.*, 1989). Nisoxetine had the same effect, so this inhibitory action is not drug-specific. The inhibition of nicotine-evoked secretion but not secretion in response to electrical stimulation suggests an interaction of these $uptake_1$ blockers with presynaptic cholinergic receptors. It is unlikely that this is the mechanism of inhibition in chromaffin cells, however, since the response to metabolic inhibitors is not associated with receptor-mediated secretion. Regardless of the mechanism by which desipramine

inhibits catecholamine release evoked by nicotine or metabolic inhibition it follows that desipramine cannot be used to differentiate between exocytosis and carrier-mediated efflux in the chromaffin cell or in the perfused heart.

To overcome this problem the effect of the two isomers of oxaprotiline on chromaffin cells (Fig 4.9) and on perfused rat hearts (Figs 4.10 and 4.11) was examined. Oxaprotiline did not inhibit catecholamine release produced by metabolic inhibition in chromaffin cells. There was a significant reduction in noradrenaline overflow in response to 40min stop-flow ischaemia in the rat heart in the presence of (+) but not (-) oxaprotiline. It should be noted that the magnitude of noradrenaline overflow in response to ischaemia was considerably lower (117 ± 21 pmol/g) than that previously reported by other workers (for example, Schömig *et al.*, 1984, reported an overflow of 817 ± 110 pmol/g after 40min ischaemia). The model of ischaemia used by Schömig *et al.* (1984) was one of isolated perfusion of the rat heart, whereby the heart is removed from the chest cavity, the aorta cannulated and the heart maintained in a humidified, warmed perfusion chamber. In the model of ischaemia used in the present work hearts were not excised. It is possible that the difference in noradrenaline overflow is due to modulation of release by the sympathetic nerve supply, as this is still intact. The studies of Schömig *et al.* (1984) were, however, performed on Wistar rats whereas the present experiments were carried carried out on Sprague Dawley rats so the differences observed may be due to strain variation.

Catecholamine release from isolated chromaffin cells in response to metabolic inhibition possesses several features that suggest it is distinct from carrier-mediated efflux of noradrenaline observed in the rat heart. Catecholamine release is no greater than that observed in response to other secretagogues. A much greater release was anticipated from the magnitude of noradrenaline overflow in ischaemic rat hearts. Furthermore, although release is calcium-independent it is not enhanced in the absence of calcium, in contrast to predictions from the model of neuronal sodium homeostasis and non-exocytotic, calcium-independent noradrenaline release proposed by Schömig *et al.* (1988). Finally, catecholamine release was not inhibited by (+) oxaprotiline at a concentration shown to inhibit noradrenaline overflow in the ischaemic rat heart and to block the uptake carrier by almost 100%. These observations suggest that the mechanism of catecholamine release is distinct from that observed in the anoxic rat heart and must occur by a mechanism other than carrier-mediated

efflux.

Chapter Five

Role of Na^+ in Catecholamine Release and Examination of the Reversibility of the Uptake Carrier in Chromaffin Cells

5.1 Introduction

The catecholamine release described in Chapter 4. could not be distinguished from an exocytotic mode of release by its sensitivity to uptake blockade. In order to elucidate the mechanism of release evoked by conditions of metabolic inhibition the role of extracellular sodium was investigated since sodium influx is central to both exocytosis in chromaffin cells (Amy and Kirshner, 1982) and carrier-mediated efflux in the ischaemic myocardium (Schömig *et al.*, 1988). Resting concentrations of cytosolic sodium are maintained at a low level primarily by the ATP-dependent Na^+/K^+ pump. Stimulation of chromaffin cells with a range of secretagogues results in a rapid influx of sodium which may be involved in mediating catecholamine secretion, as demonstrated by Amy and Kirshner (1982). Nicotine-stimulated catecholamine secretion was accompanied by a marked uptake of $^{22}Na^+$. Secretion was not, however, completely dependent on extracellular Na^+ as long as Ca^{2+} was present. In contrast, other workers have reported reduced catecholamine release in the absence of external sodium (Wada *et al.*, 1984). It seems that, where nicotinic stimulation is maximal, this alone is sufficient to trigger calcium influx and evoke secretion. When nicotinic stimulation, however, is submaximal sodium influx via receptor-mediated sodium channels may facilitate calcium influx. Nicotinic secretion is thus partially dependent on external sodium under certain conditions.

Veratridine-stimulated catecholamine secretion is completely abolished by removal of external sodium since, in this case, catecholamine secretion is triggered by direct depolarisation of the plasma membrane following Na^+ influx via voltage-sensitive Na^+-channels. Thus under certain conditions Na^+ influx via receptor-linked channels or voltage-sensitive channels; or both, may be a prerequisite for secretion in chromaffin cells, probably by facilitating the subsequent influx of calcium.

Ischaemia-induced noradrenaline overflow in the heart requires an elevated cytosolic catecholamine concentration and a reduction of the inwardly directed sodium gradient. Noradrenaline efflux will therefore be triggered following an influx of sodium to raise cytosolic sodium levels or by reversal of the sodium gradient by removal of external sodium. The role of sodium in noradrenaline overflow induced by ischaemia, anoxia or cyanide intoxication in rat hearts was investigated by Schömig *et al.* (1988). Sodium fluxes via different pathways were modulated using selective inhibitors and the resultant effect on

noradrenaline overflow studied. A raised cytosolic sodium concentration caused an efflux of noradrenaline only when cytosolic amine concentrations were also artificially elevated. In the presence of reserpine to deplete vesicular amine stores inhibition of the Na^+/K^+ ATPase with ouabain or stimulation of sodium influx with veratridine mediated a rapid efflux of noradrenaline. This use of pharmacological inhibitors demonstrated that carrier-mediated efflux was a plausible mechanism for release from cardiac sympathetic neurons.

There is evidence for intracellular sodium accumulation in myocytes (Fiolet *et al.*, 1984) but no direct evidence in sympathetic neurons under pathophysiological conditions, although pharmacological evidence suggests that sodium influx is involved in ischaemia-induced efflux. Thus, this overflow was inhibited by amiloride and ethylisopropyl-amiloride (EIPA) but not by tetrodotoxin, indicating that sodium influx via the Na^+/H^+ exchanger is the predominant route for sodium entry.

In contrast, noradrenaline release in response to cyanide intoxication was not inhibited by EIPA but was reduced by tetrodotoxin (Schömig *et al.*, 1988). It was suggested that intracellular acidosis under conditions of ischaemia would inhibit sodium influx through tetrodotoxin-sensitive channels whereas the Na^+/H^+ exchanger would be activated under these conditions (i.e reduced cytosolic pH). During cyanide intoxication, in contrast, there was continuous washout of metabolites, reducing intracellular acidosis, so that the tetrodotoxin-sensitive Na^+ channels appeared to be the major route for Na^+ entry.

The role of sodium in relation to catecholamine release under conditions of metabolic inhibition was initially investigated to determine whether there was a sodium influx as predicted from the work of Schömig *et al.* (1988). However, once it became evident that the mode of catecholamine release differed from that in the myocardium it was hoped that comparison of the influx of sodium with that observed during exocytosis might provide some indication of the mechanism of the calcium-independent catecholamine release. In addition, pharmacological agents were utilised to attempt to induce carrier-mediated efflux under conditions demonstrated to be effective in cardiac sympathetic neurons (Schömig *et al.*, 1988) and in PC12 cells (Bönisch *et al.*, 1984).

5.2 Results

5.2.1 $^{22}Na^+$ influx and catecholamine secretion.

Sodium influx was measured in parallel with catecholamine release using a

number of different agents known to promote $^{22}Na^+$ uptake (Fig 5.1). Amy and Kirshner (1982) previously reported an increase in $^{22}Na^+$ uptake within 10min in the presence of 10μM nicotine or 100μM veratridine. They found values of 8.3 and 14.2nmol $^{22}Na^+$/well/10min (27.7 and 47.3 nmol/10^6cells); and 23.2 and 34.4% catecholamine secretion in the presence of 10μM nicotine and 100μM veratridine respectively. Similar results were obtained here, 14 and 19.5 nmol $^{22}Na^+$/well/10min (28 and 39nmol/10^6cells) and 18.7 and 20.5% catecholamine release being found. The catecholamine secretion in response to 100μM veratridine may be lower than that reported by Amy and Kirshner (1982) since veratridine-stimulated secretion was maximal at 50mM Na^+ (Na^+ level present in their study) rather than at 154mM Na^+ (the Na^+ concentration used in these experiments).

Monensin, a monovalent cation ionophore, also induces Na^+-dependent catecholamine secretion from chromaffin cells (Suchard *et al.*, 1982). However, catecholamine release and $^{22}Na^+$ influx are not observed until at least one hour after exposure to the ionophore. In this study, catecholamine release and $^{22}Na^+$ influx above basal levels were seen after 1h exposure.

Like the secretagogues, metabolic inhibition caused an influx of $^{22}Na^+$ within 10min as with catecholamine release, suggesting a possible involvement of Na^+ in catecholamine release under these conditions.

5.2.2 Effect of Na^+ concentration on $^{22}Na^+$ uptake and catecholamine release.

Fig 5.2 illustrates the effect of altering the external Na^+ concentration on catecholamine release and $^{22}Na^+$ influx in response to 10μM nicotine (A) and metabolic inhibition (B) respectively. $^{22}Na^+$ uptake in the presence of 10μM nicotine increased proportionally with increasing Na^+ concentration. Nicotine-stimulated catecholamine release was much less dependent on extracellular sodium as found previously by Amy and Kirshner (1982). Catecholamine secretion was partially reduced at low concentrations of Na^+, being 36% lower at 10mM Na^+ than at 154mM Na^+.

Catecholamine release in response to metabolic inhibition was markedly reduced at Na^+ levels below 75mM. There was no influx of $^{22}Na^+$ above basal levels until the external Na^+ concentration reached 75mM. This suggests that Na^+ influx is coupled to catecholamine release. Release may not occur until intracellular Na^+ concentrations reach a certain level or are high enough to mediate a secondary response that is the immediate trigger. Both veratridine and monensin-induced

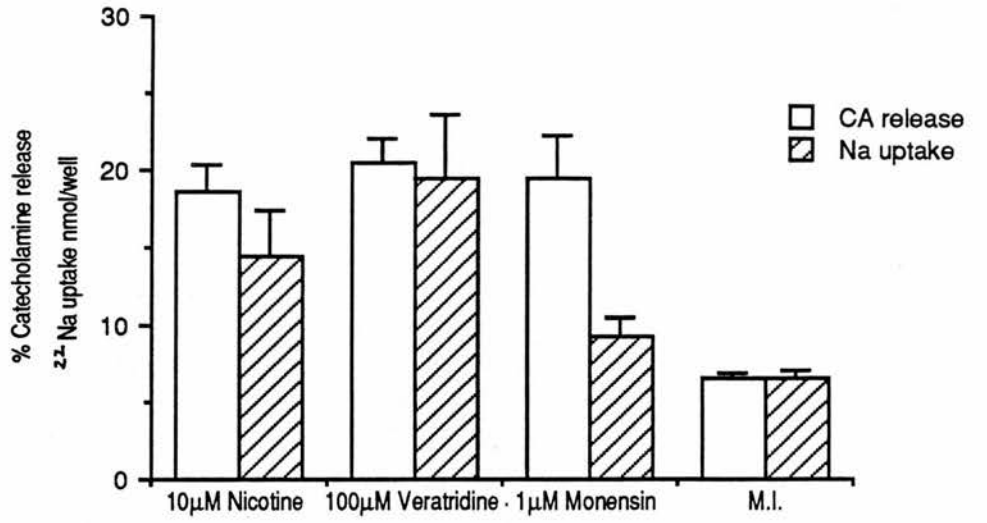


Fig 5.1 Catecholamine release and uptake of $^{22}Na^{+}$. Cells were treated with Locke's buffer or Locke's buffer plus 10μM nicotine, 100μM veratridine or metabolic inhibitors for 10min; or 1μM monensin for 60min. Results are expressed as percentage catecholamine release, or $^{22}Na^{+}$ uptake, nmol/well ($0.5x10^{6}$ cells/well), after subtraction of basal levels, means ± SE of triplicate determinations.

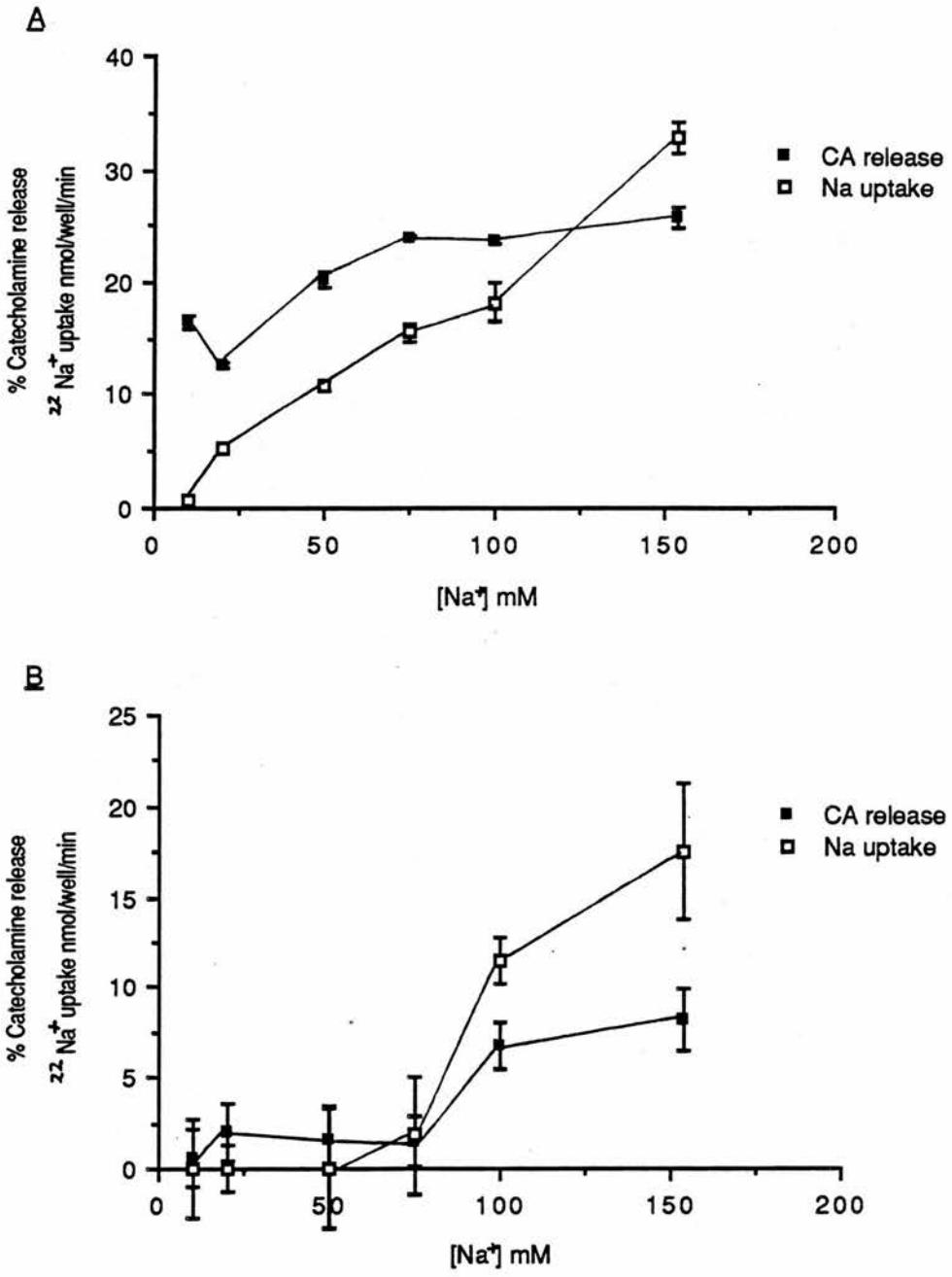


Fig 5.2 Catecholamine release and $^{22}Na^{+}$ influx as a function of extracellular Na^{+} concentration. Cells were treated with Locke's buffer or Locke's buffer containing reduced Na^{+} was adjusted with sucrose to maintain osmosity. Results are expressed as percentage catecholamine release or $^{22}Na^{+}$ uptake (nmol/well/10min) above basal, means ± SE of triplicate determinations. A response to nicotine, B response to metabolic inhibition.

catecholamine secretion are also dependent on external sodium.
Veratridine-stimulated secretion declined sharply at Na^+ levels below 25mM (Amy and Kirshner, 1982) and monensin-induced secretion was reduced by 30% at 10mM Na^+ (Suchard *et al.*, 1982).
In strong contrast, noradrenaline efflux in the anoxic myocardium was greater when external sodium was reduced and was replaced with either LiCl, Tris or sucrose (Dart and Riemersma, 1989). This was in keeping with the proposed model of carrier-mediated efflux (Schömig *et al.*, 1988), dependent on reversal of the inwardly directed sodium gradient to an outwardly directed gradient.

5.2.3 Effect of inhibitors of Na^+ entry on catecholamine release.

The effect of a range of inhibitors of different Na^+ channels on catecholamine release under conditions of nicotine-stimulation and metabolic inhibition was investigated. $^{22}Na^+$ influx following nicotine-stimulation is sensitive to hexamethonium which blocks nicotinic receptor-linked Na^+ channels, but not to inhibitors of voltage-sensitive Na^+ channels such as tetrodotoxin (Wada *et al.*, 1984).
Noradrenaline overflow in the heart induced by ischaemia (Schömig *et al.*, 1988) or substrate-free anoxia (Dart and Riemersma, 1989) was significantly reduced by amiloride and EIPA, inhibitors of the Na^+/H^+ exchanger, but not by tetrodotoxin or lignocaine, both inhibitors of voltage-sensitive Na^+ channels. Amiloride inhibits the Na^+/H^+ exchange system competitively so that high (millimolar) concentrations of amiloride are required to block the Na^+/H^+ exchanger at physiological sodium concentrations. Near maximal inhibition of the exchanger has been reported at 0.1mM amiloride in 3T3 cells, chick skeletal muscle cells and chick cardiac cells (Vigne *et al.*, 1983). Fig 5.3 demonstrates the sensitivity of catecholamine release (A) and $^{22}Na^+$ uptake (B) to 0.1mM amiloride. $^{22}Na^+$ influx in response to nicotine-stimulation was significantly reduced in the presence of 0.1mM amiloride ($p<0.001$). There was a parallel reduction of catecholamine release. Unlike ischaemia-induced noradrenaline overflow from the perfused rat heart, catecholamine release induced by metabolic inhibition was not inhibited by 0.1mM amiloride ($p>0.5$) nor was there any reduction in $^{22}Na^+$ influx.
Amiloride is known not to be specific for the Na^+/H^+ exchanger, inhibiting the Na^+/ Ca^{2+} exchanger and the Na^+/K^+ ATPase in some systems (Lazdunski *et al.*, 1985). Consequently the action of EIPA, a potent but more specific analog of

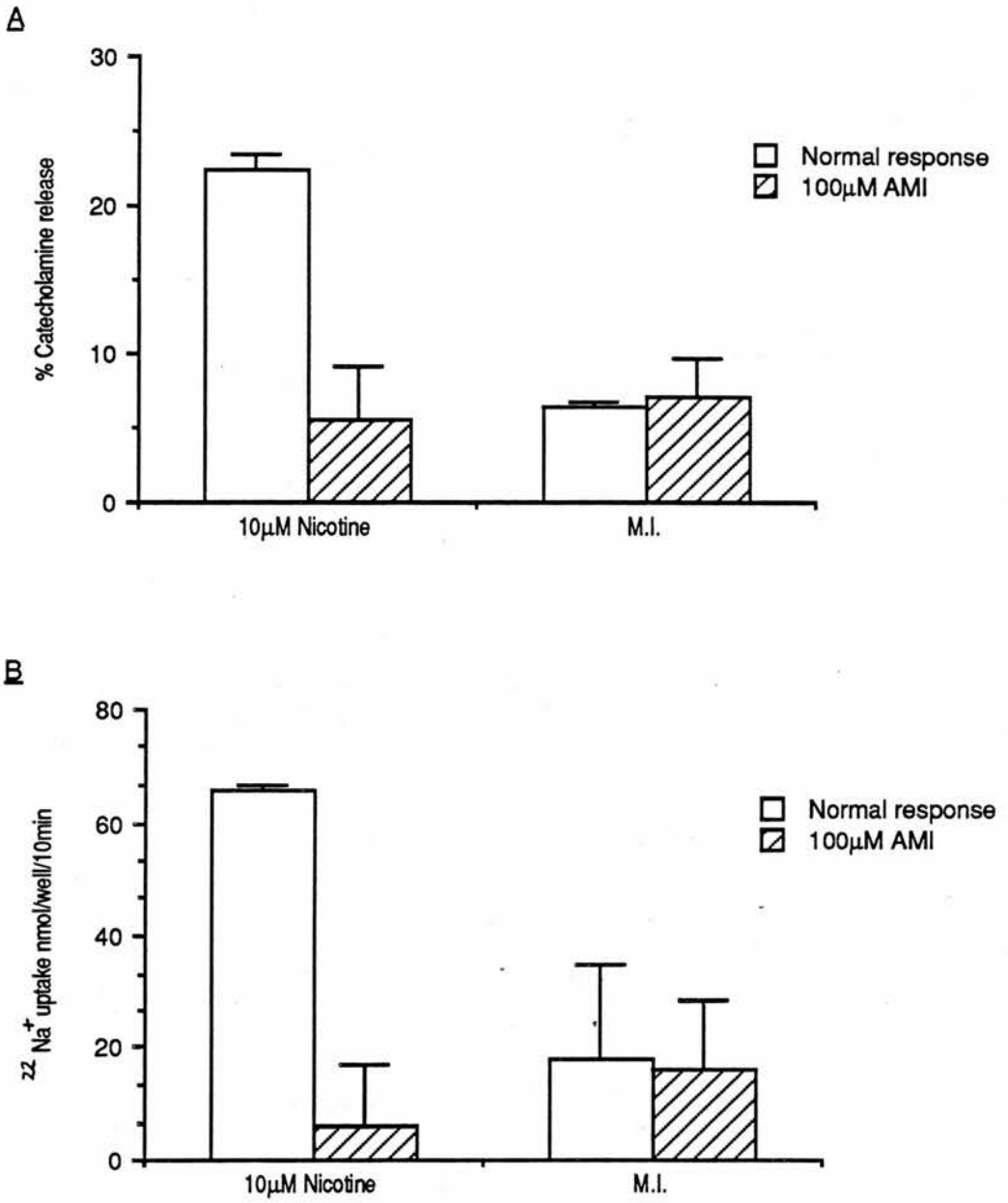


Fig 5.3 Sensitivity of catecholamine release and uptake of $^{22}Na^{+}$ to amiloride. Catecholamine release and $^{22}Na^{+}$ uptake were measured after 10min treatment with Locke's buffer plus 10μM nicotine or metabolic inhibitors in the presence or absence (normal response) of 100μM amiloride (AMI). Results are expressed as percentage catecholamine release (A) or $^{22}Na^{+}$ uptake nmol/well/10min (B) above basal levels, means ± SE of triplicate determinations.

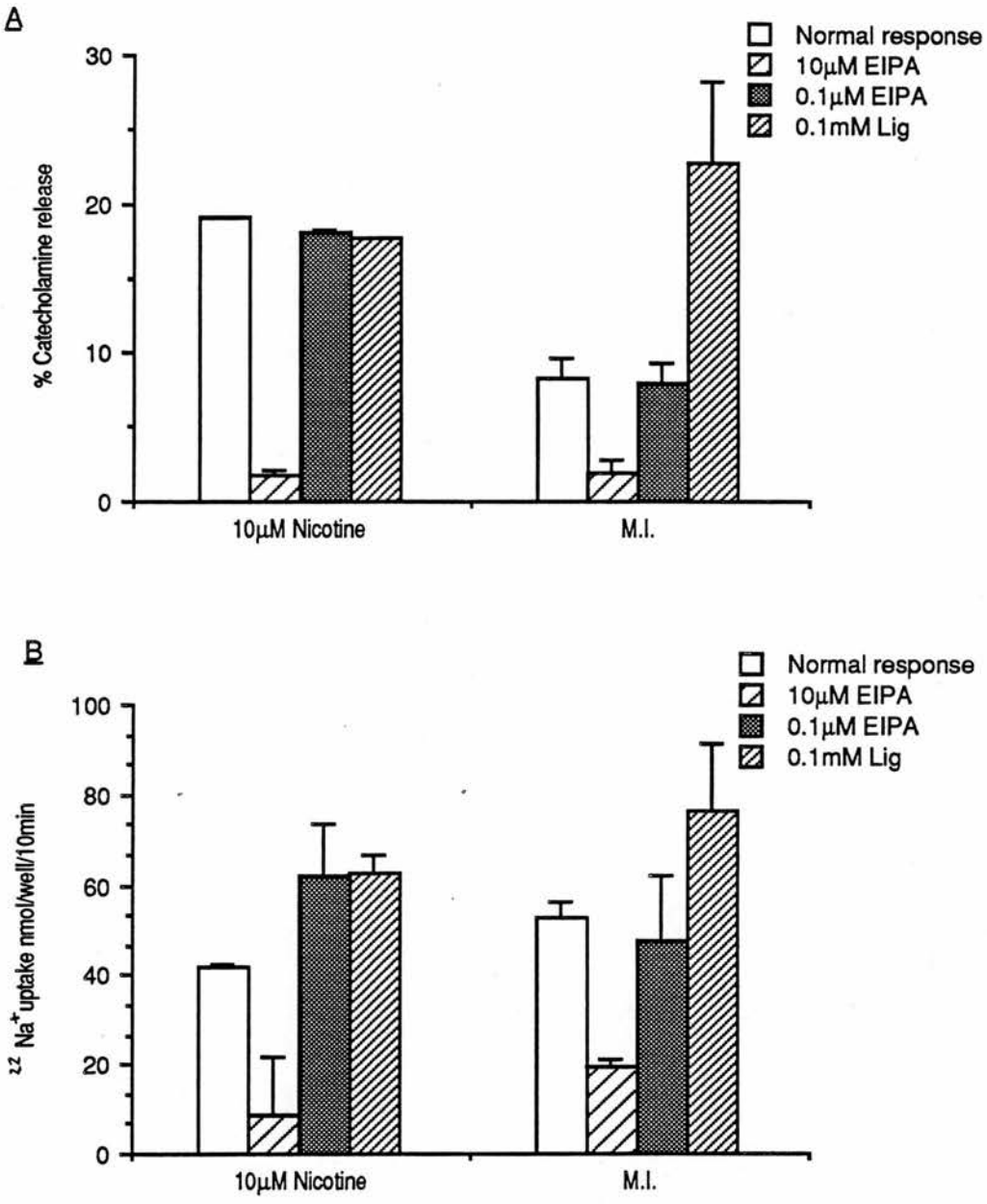


Fig 5.4 Sensitivity of catecholamine release and uptake of $^{22}Na^{+}$ to EIPA or lignocaine. Percentage catecholamine release and $^{22}Na^{+}$ influx nmol/well/10min were measured after 10min in the presence or absence (normal response) of 0.1 and 10μM EIPA or 0.1mM lignocaine (Lig). Results above basal levels are means ± SE of triplicate determinations.

amiloride (Vigne *et al.*, 1983) was also examined (Fig 5.4). 10μM EIPA significantly reduced catecholamine release (A) and $^{22}Na^+$ influx (B), $p<0.001$, under conditions of both nicotine-stimulation and metabolic inhibition. 0.1μM EIPA had no inhibitory effect on catecholamine release under these conditions. Influx of $^{22}Na^+$ appeared to be potentiated, but this was not significant ($p>0.5$). Lignocaine, which inhibits voltage-sensitive Na^+ channels, did not significantly alter either catecholamine release or $^{22}Na^+$ influx in response to nicotine (Fig 5.4). Both, however, were markedly increased ($p<0.001$) by 0.1mM lignocaine in the presence of metabolic inhibitors (Fig 5.4).

5.2.4 Reversibility of the chromaffin cell Na^+ /catecholamine symporter.

The imposition of anoxic conditions in chromaffin cells did not induce a release of catecholamines that demonstrated any of the features predicted for carrier-mediated efflux. This raised the question of whether a carrier-reversal mechanism could operate in chromaffin cells. Experiments were based on those performed by Bönisch *et al.* (1984) on noradrenaline transport in PC12 cells. In this work, PC12 cells were loaded with [^{3}H] noradrenaline in the presence of reserpine, to prevent vesicular uptake, and pargyline, to inhibit MAO-catalysed oxidation of the noradrenaline accumulating in the cytosol. When these cells were then exposed to Na^+-free conditions there was a marked efflux of [^{3}H] noradrenaline via the uptake carrier, demonstrating that this carrier, like that in adrenergic nerve terminals (Paton, 1973) is bidirectional.

Chromaffin cells were treated with 0.5μM reserpine plus 0.1mM pargyline for 24h. Previous studies on reserpine-treated cells showed that the cellular catecholamine content was reduced by approximately 50% under these conditions (Caughey and Kirshner, 1987). It should be noted that at the dilution used DMSO, the solvent for reserpine, did not affect the ability of the cells to respond normally to nicotine or high K^+. In the presence of pargyline it is expected that cytosolic catecholamines will increase in concentration, but will not be metabolised.

Following reserpine treatment the total cellular catecholamine content was found to be depleted by 36% (in the absence of pargyline). Fig 5.5 confirms the results of Caughey and Kirshner that prolonged reserpine treatment reduced but did not inhibit the secretory response. Exposure of reserpine-treated cells to Na^+-free buffer (Na^+ replaced with sucrose to maintain osmosity) did not result in any significant extra release of catecholamines compared to that released from

untreated cells in the presence of 0.1mM pargyline only (Fig 5.5).

In order to demonstrate that the cytosolic catecholamine concentration is indeed elevated following reserpine-treatment, despite this lack of efflux, cells were treated for 10min with 10μM digitonin. Digitonin selectively permeabilises the plasma membrane of chromaffin cells, enabling direct stimulation of secretion on the addition of micromolar levels of calcium (Dunn and Holz, 1983). This is confirmed in the experiment shown in Fig 5.6, the response of cells to readdition of calcium following 10min permeabilisation with 10μM digitonin being shown. There was a catecholamine release of 18.3% in the presence of calcium compared with only 9.4% in the absence of calcium. When reserpine-treated cells were permeabilised with digitonin 9.9% catecholamines were released compared with 2.6% released from untreated cells (Fig 5.5), suggesting that reserpine treatment has indeed elevated the cytosolic concentration of catecholamines. Calcium-free buffer alone, in the absence of digitonin, did not release catecholamines much above basal levels (Fig 5.5). These results suggest that, unlike previous assumptions concerning the similarity of the chromaffin cell carrier to that in adrenergic neurons, the chromaffin cell uptake carrier is not reversible under conditions of elevated cytosolic catecholamines, and reduced extracellular Na^+.

Bönisch *et al.* (1984) loaded PC12 cells with [^3H] noradrenaline in the presence of reserpine in order to study noradrenaline transport. In order to make a direct comparison with their experiments chromaffin cells were incubated with [^3H] noradrenaline in the presence of 0.5μM reserpine and 0.1mM pargyline for 24h prior to permeabilisation with digitonin or exposure to Na^+-free conditions. As in the previous study (Fig 5.5) Na^+-free conditions did not cause release of [^3H] noradrenaline following reserpine pretreatment (Fig 5.7). [^3H] noradrenaline was, however, sequestered in the cytosol in the presence of reserpine as demonstrated by its release from the cells on permeabilisation with digitonin.

Tyramine, a sympathomimetic amine, is taken up into isolated chromaffin granules and displaces endogenous catecholamines, possibly by disruption of the granule electrochemical proton gradient (Johnson *et al.*, 1982). Chromaffin cells take up tyramine via a cocaine sensitive mechanism (Youdim *et al.*, 1986) and tyramine has been reported to displace catecholamines from whole adrenal glands from pigs (Nakai and Yamada, 1983) but not from rats (Wakade and Wakade, 1984). It was postulated that tyramine, like reserpine, would displace

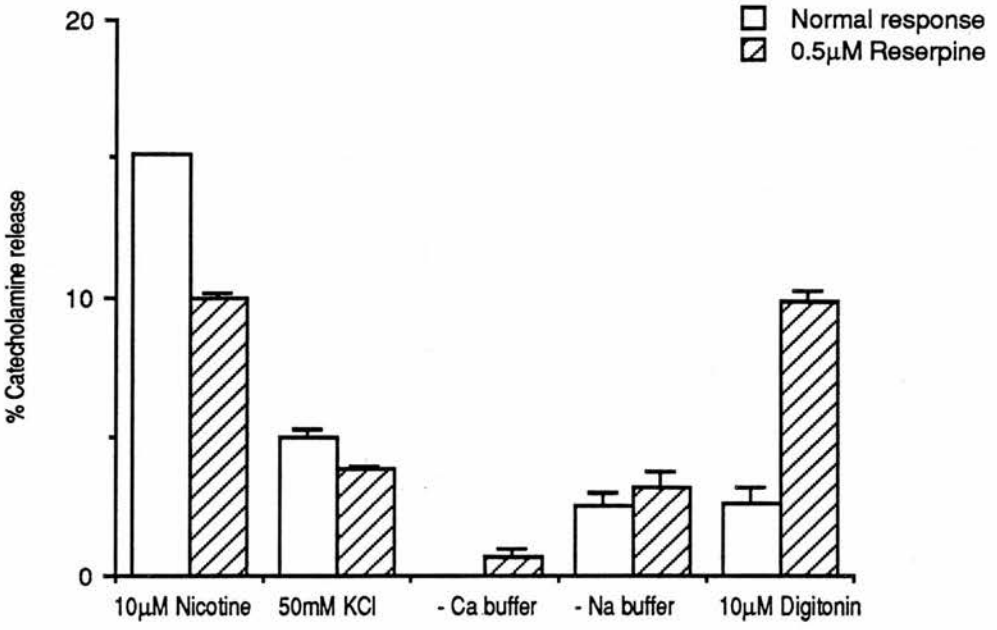


Fig 5.5 Response to Na^+-free conditions after depletion of granule catecholamine stores with reserpine. Cells were subjected to 0.5μM reserpine plus 0.1mM pargyline for 24h in culture medium. They were then exposed to Locke's buffer plus 10μM nicotine or 50mM KCl for 10min. Where Na^+-free Locke's was used, $Na^+$was replaced with sucrose. Cells were also exposed to Ca^{2+}-free buffer plus or minus 10μM digitonin for 10min. Results show percentage catecholamine release evoked by these conditions on treated (0.5μM reserpine) and untreated (normal response) cells, above basal levels, means ± SE of triplicate determinations.

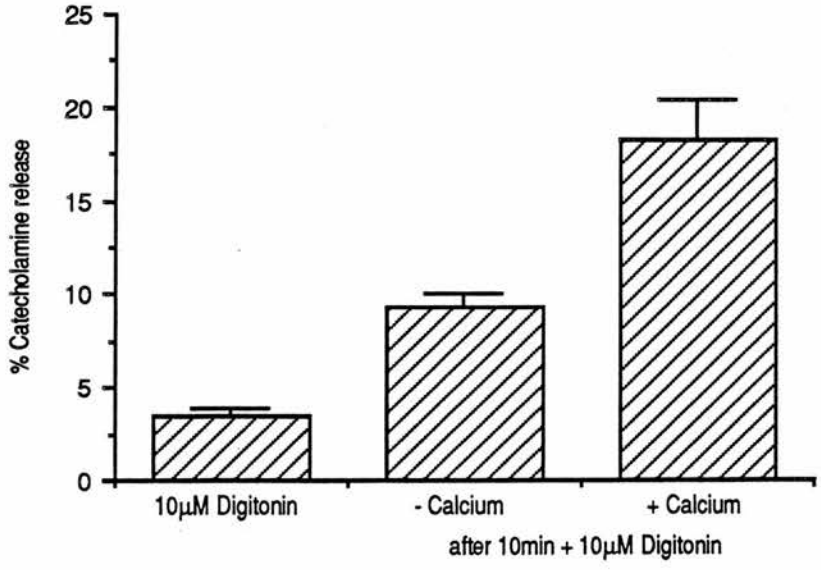


Fig 5.6 Response to extracellular calcium following permeabilisation with 10μM digitonin. Cells were permeabilised for 10min with 10μM digitonin in Ca^{2+}-free buffer. They were then exposed to buffer+4.43mM Ca^{2+} or minus Ca^{2+} for a further 10min. Results are expressed as percentage catecholamine release, means ± SE of triplicate determinations.

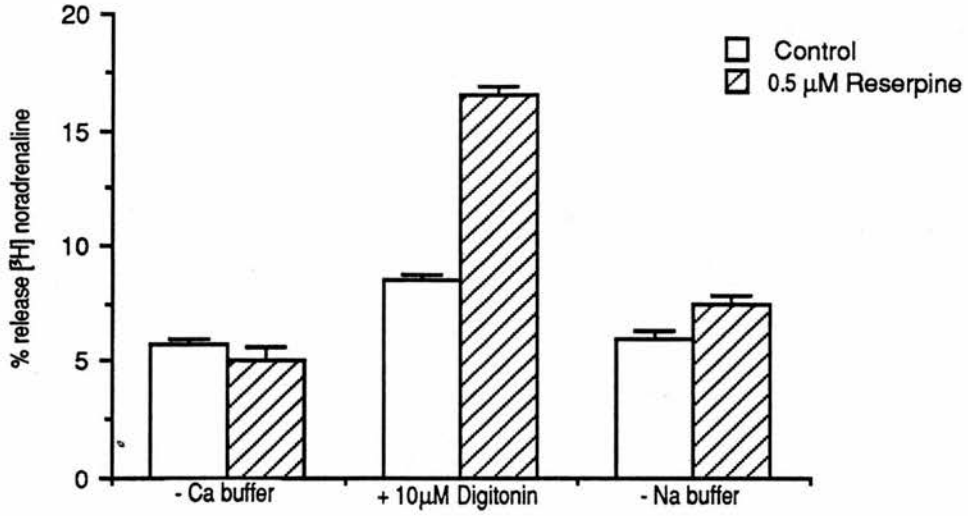


Fig 5.7 Response to Na^+-free conditions after depletion of endogenous catecholamines and labelling of the cytosolic pool with [^{3}H] noradrenaline. Cells were incubated for 24h with 0.5µM reserpine plus 0.1mM pargyline and [^{3}H] noradrenaline in DMEM (conditions as previously described for uptake of [^{3}H] noradrenaline, Chapter 2.). Cells were then treated with Ca^{2+}-free buffer, Ca^{2+}-free buffer plus 10µM digitonin, or Na^+-free buffer for 10min. Results are expressed as percentage release of [H] noradrenaline from untreated (control) cells or reserpine-treated (0.5µM reserpine) cells, means ± SE of triplicate determinations.

catecholamines from isolated chromaffin cells and elevate cytosolic catecholamine concentrations. Exposure of chromaffin cells to 0.1mM tyramine for up to 4h caused no reduction in total cell catecholamine content, nor did this treatment induce any increase in release following permeabilisation with digitonin (results not shown). This lack of tyramine effect in chromaffin cells has previously been reported by Youdim *et al.* (1986) but in their work chromaffin cells were only treated with tyramine for 15min. PC12 cells, however, did demonstrate a tyramine releasable pool of catecholamines within this time (Youdim *et al.*, 1986).

5.3 Discussion.

Stimulation of catecholamine secretion by nicotine or by the voltage-dependent Na^+ channel activator veratridine, was accompanied by an increase in cellular uptake of $^{22}Na^+$ (Fig 5.1). These observations were similar to those previously reported by Amy and Kirshner (1982). Nicotine-stimulated release is partially dependent on extracellular sodium, secretion being inhibited by 36% at 10mM Na^+ (fig 5.2A). This has been attributed to the ability of the cells to elicit a Ca^{2+} influx independently of activation of the voltage-dependent Na^+ channels (Marley, 1988). The sensitivity of nicotine-evoked secretion to extracellular Na^+ seems to vary with different cell preparations (personal observations), and may explain the Na^+-independent release reported by Amy and Kirshner (1982) compared with the Na^+-dependence of nicotine-evoked release reported by Wada *et al.* (1984).

Na^+ influx in response to nicotine-stimulation was not inhibited by the voltage-dependent Na^+ channel inhibitor, lignocaine (Fig 5.4A). This confirms the observations of Wada *et al.* (1984) that stimulation with nicotine causes an influx of Na^+ through receptor-linked but not through voltage-dependent Na^+ channels. The Na^+/H^+ exchange inhibitor, amiloride, blocked both catecholamine secretion and Na^+ influx in response to nicotine stimulation (Fig 5.3A and B). A similar result was seen with its more potent analog, EIPA, at a concentration of 10μM but not at 0.1μM. There have been few reports on the effect of amiloride on chromaffin cells. Friedman *et al.* (1986) reported that heavy meromyosin could stimulate catecholamine secretion, that this was dependent on extracellular Na^+, and that $^{22}Na^+$ uptake caused by heavy meromyosin was inhibited by 10μM amiloride. Acetylcholine-stimulated secretion and $^{22}Na^+$ uptake were both enhanced by the incorporation of heavy meromyosin into chromaffin cells using

lipofection. In conjunction with these observations they reported that 100μM amiloride almost abolished catecholamine secretion induced by both heavy meromyosin and acetylcholine, in agreement with the observations reported here (Fig 5.3).

Amiloride is a competitive inhibitor of the Na^+/H^+ exchanger and is only effective at high concentrations. The K_i for amiloride, as measured in chromaffin granule membranes, was 0.26mM (Haigh and Phillips, 1989). Consequently at a Na^+ concentration of 154mM, 100μM amiloride will not be sufficient to inhibit Na^+/H^+ exchange. The inhibition of the nicotinic response is, therefore, probably due to a non-specific effect of the drug acting at the nicotinic receptor-linked Na^+ channel.

EIPA is approximately 1000x more potent than amiloride and also highly specific for the Na^+/H^+ exchanger, not having the non-specific action on other ion channels that are seen with amiloride. EIPA has a K_i value of 0.15μM at 140mM Na^+ (Lazdunski *et al.*, 1985). At a concentration of 10μM, EIPA may have inhibitory action other than inhibition of the Na^+/H^+ exchanger. Inhibition of the nicotinic response with 10μM EIPA may be due to a similar effect as that seen with amiloride. No inhibition of $^{22}Na^+$ uptake was seen with 0.1μM EIPA when the drug will be totally selective for the Na^+/H^+ exchanger. The Na^+/H^+ exchanger is unlikely, therefore, to play a role in stimulus-secretion coupling in chromaffin cells.

Metabolic inhibition, like nicotine-stimulation, caused an influx of $^{22}Na^+$, associated with catecholamine secretion (Fig 5.1). Unlike the nicotinic response, however, this $^{22}Na^+$ uptake showed a marked dependence on extracellular Na^+ concentration. Whereas veratridine-induced $^{22}Na^+$ uptake was proportional to the Na^+ concentration in the external media (Amy and Kirshner, 1982), metabolic inhibition did not produce any significant $^{22}Na^+$ influx over 10min until an external Na^+ concentration of more than 75mM was reached (Fig 5.2B). Catecholamine secretion exhibited a parallel sensitivity to the external sodium concentration.

The Na^+ influx also differed from that evoked by veratridine in that it was not inhibited by blockers of voltage-dependent Na^+ channels, in this case lignocaine (Fig 5.4B). In fact both catecholamine release and Na^+ influx caused by metabolic inhibition were significantly enhanced by 0.1mM lignocaine. The reason for this

enhanced response is not known though it is striking that both increase in parallel. It is possible that inhibition of the voltage-dependent Na^+ channels activates Na^+ influx and thus catecholamine release through some other route. 10mM EIPA but not 0.1μM EIPA or 0.1mM amiloride inhibited $^{22}Na^+$ influx and catecholamine release caused by metabolic inhibition. Whilst it is clear that catecholamine release under these conditions has an absolute requirement for external Na^+, and this must attain a certain level before release is promoted, the discrepancy in the actions of amiloride and EIPA make it difficult to assess the route of Na^+ entry. Possible mechanisms for Na^+-dependent catecholamine release have, however, been suggested from the action of other pharmacological agents on chromaffin cells.

5.3.1 Na^+ influx and catecholamine release evoked by other pharmacological agents.

A Monensin

Prolonged exposure to 1μM monensin, an ionophore for monovalent cations, resulted in catecholamine release and $^{22}Na^+$ influx in chromaffin cells (Fig 5.1). Exposure of isolated rat hearts to monensin for 10min did not cause an overflow of noradrenaline (Schömig *et al.*, 1988). There was a large overflow of DOPEG, however, indicative of a large cytoplasmic noradrenaline concentration, released by disruption of the vesicular proton gradient by monensin. In the rat heart monensin did not promote a neuronal sodium influx until concentrations higher than 10μM were used (Schömig *et al.*, 1988). The response of chromaffin cells to 1μM monensin is linear for at least 6h (Suchard *et al.*, 1982) and observations in this study (results not shown). Monensin-induced catecholamine release was Na^+-dependent but Ca^{2+}-independent (Suchard *et al.*, 1982), as is true for catecholamine release caused by metabolic inhibition (Fig 5.2B and 4.5) but clearly differing from noradrenaline overflow observed in the rat heart (Dart and Riemersma, 1989). From these observations , Suchard *et al.* (1982) postulated that the monensin-mediated rise in intracellular Na^+ raises intracellular Ca^{2+} independently of extracellular Ca^{2+}, and that this , in turn, stimulates exocytosis. This Na^+-dependent, Ca^{2+}-independent catecholamine release has also been reported by Izumi *et al.* (1986). Release of catecholamines in their study was not, however, accompanied by release of DBH, which is co-released with catecholamines during exocytosis. Monensin also caused an influx of $^{22}Na^+$ into isolated chromaffin granules. The consequent Na^+-dependent catecholamine loss

was accompanied by loss of DBH, suggesting granule lysis. Lysis of isolated chromaffin granules and granules in intact cells evoked by monensin has previously been reported by Geisow and Burgoyne (1982). This would result in a rise in cytoplasmic catecholamine. Only catecholamines are released from the cell suggesting a non-exocytotic mechanism of release that is Ca^{2+}-independent (Izumi *et al.*, 1986). This release may be carrier-mediated. However, it was inhibited by low external Na^+, unlike anoxia-induced release that is enhanced by reduction of external Na^+.

B Ouabain.

The cardiac glycoside, ouabain, which inhibits the Na^+/K^+ ATPase, also triggers catecholamine release from chromaffin cells (Suchard *et al.*, 1982). The time course for catecholamine release is essentially the same as that evoked by monensin. Ouabain causes an elevation of intracellular Na^+ which is thought to mediate catecholamine release by elevating intracellular calcium. Pocock (1983a and b) studied the possible mechanism of action of ouabain extensively. Ouabain-evoked secretion was shown to be exocytotic by the parallel release of DBH but not lactate dehydrogenase. The catecholamine secretion had an absolute requirement for extracellular sodium and was nominally independent of extracellular calcium. Török and Powis (1988) have reported that ouabain evoked catecholamine release from chromaffin cells was totally Ca^{2+}-dependent. A further discrepancy has been observed in the sensitivity of ouabain-stimulated secretion to tetrodotoxin (Pocock, 1983a; and Powis *et al.*, 1989).

Pocock (1983b) suggested that ouabain elicits release through a direct inhibitory effect on the calcium-extrusion mechanism, leading to a rise in the resting level of cytosolic calcium. In the absence of extracellular sodium, calcium efflux is not inhibited by ouabain and this would account for the sodium-dependence of release. This catecholamine release is inhibited by cocaine and desipramine at concentrations which block catecholamine uptake (Powis *et al.*, 1989). This has also been reported by Sweadner (1985) in rat sympathetic neurons, ouabain-evoked release being sensitive to desipramine, Na^+-dependent and Ca^{2+}-independent. Consequently a mechanism of carrier-mediated efflux was proposed. However, such a mechanism of release would be expected to be stimulated in the absence of extracellular sodium rather than inhibited. This mechanism of release has been discounted in chromaffin cells in response to ouabain for this very reason (Powis *et al.*, 1989). These authors suggest that

ouabain-mediated Na^+ accumulation leads to a Ca^{2+} influx since this Na^+ accumulation itself may cause depolarisation and opening of the tetrodotoxin-sensitive Na^+ channels. This in turn would lead to further Na^+ accumulation and enhance Ca^{2+} influx, and subsequent catecholamine release by exocytosis.

Such a mechanism may also be attributed to the release of [^3H] noradrenaline in sympathetic neurons reported by Sweadner (1985). However, the possibility that Na^+/K^+ ATPases in different tissues respond differently to ouabain cannot be discounted. Furthermore, the different observations on ouabain-evoked catecholamine release in chromaffin cells discussed here suggest that the mode of action of this glycoside has yet to be completely elucidated.

5.3.2 Na^+ influx and catecholamine release caused by metabolic inhibition.

Metabolic inhibition caused a similar Na^+-dependent catecholamine release to that evoked by ouabain. Metabolic inhibition, like ouabain, would be expected to cause a rapid inhibition of Na^+/K^+ ATPase activity. Such an effect was reported by Pocock (1983b). Anoxic chromaffin cells quickly lost their ability to transport sodium and potassium ions and there was a concomitant rise in intracellular sodium. In the present study this sodium influx was observed within 10min after the onset of metabolic inhibition in conjunction with catecholamine release. In contrast, cells were exposed to ouabain for at least 60min before any significant changes were observed (Powis *et al.*, 1989). Consequently catecholamine release caused by metabolic inhibition cannot be the result of Na^+/K^+ ATPase inhibition alone.

$^{22}Na^+$ influx and catecholamine release evoked by metabolic inhibition was not inhibited by 0.1mM amiloride or 0.1μM EIPA (Fig 5.3 and 5.4). As discussed above, 0.1mM amiloride may not be sufficient to inhibit the Na^+/H^+ exchanger at an external sodium concentration of 154mM. In retrospect 1mM amiloride should have been used to ensure Na^+/H^+ inhibition. EIPA had the same effect on the response with metabolic inhibitors as on the nicotinic response. This implies that the Na^+/H^+ exchanger is not involved in the response evoked by metabolic inhibitors. At such a high extracellular Na^+ concentration, however, Na^+ may compete out any inhibitory action of the drug, 0.1μM being lower than the reported K_i for EIPA. The Na^+-dependence of the response prevented the use of lower extracellular Na^+ concentrations. Consequently under the conditions used it is not possible to determine whether or not the Na^+/H^+ exchanger is involved in

the response caused by metabolic inhibition.

During ischaemia in the isolated rat heart the Na^+/H^+ exchanger has been identified as being the major pathway for sodium entry (Schömig *et al.*, 1988). At physiological pH_o noradrenaline overflow during substrate-free anoxic perfusion was inhibited by amiloride and EIPA (Dart and Riemersma, 1989). Under conditions of metabolic inhibition in chromaffin cells it may be postulated that the rapid rundown of cytosolic ATP would lead to an intracellular acidosis. The Na^+/H^+ exchanger would be maximally activated leading to a Na^+ influx especially as the Na^+/K^+ ATPase will be suppressed. This Na^+ influx may then mediate catecholamine release through the mobilisation of internal Ca^{2+} stores even in the absence of extracellular Ca^{2+}. As discussed above, however, it has not been possible to demonstrate such a mechanism of release by monitoring $^{22}Na^+$ influx under conditions of metabolic inhibition in chromaffin cells.

In rat neocortical brain slices veratridine stimulates a substantial release of [^{3}H] noradrenaline that is independent of extracellular Ca^{2+} (Schoffelmeer and Mulder, 1983). This release appears to be mediated by an increase in intracellular Na^+ and subsequent mobilisation of internal Ca^{2+} stores. Veratridine in the absence of Ca^{2+} has also been reported to cause a Na^+-dependent release of noradrenaline from the vas deferens (Alberts *et al.*, 1981). In chromaffin cells, however, veratridine-mediated catecholamine release is Ca^{2+}-dependent (Kilpatrick et al., 1981). Catecholamine release induced by heavy meromyosin in chromaffin cells did not appear to be Ca^{2+}-dependent (Friedman *et al.*, 1986). They suggested that the rise in intracellular Ca^{2+} required for secretion was mediated by Na^+ influx, heavy meromyosin-induced catecholamine release being Na^+-dependent and abolished by amiloride. Therefore, a Na^+-mediated catecholamine release may occur in chromaffin cells under certain conditions.

5.3.3 Carrier-mediated efflux in bovine chromaffin cells.

The occurrence of carrier-mediated efflux mechanisms in myocardial sympathetic neurons (Schömig *et al.*, 1984) and PC12 cells (Bönisch *et al.*, 1984) led to a more detailed examination of carrier-reversibility in chromaffin cells.

Reserpine was used to inhibit the catecholamine/H^+ exchanger of the granule membrane and raise cytosolic catecholamines in the presence of pargyline, to inhibit metabolism. Following treatment with 0.5μM reserpine, permeabilisation with 10μM digitonin released 10.3% total catecholamines compared with 2.6%

released from untreated cells. This increase of 6.9% can be assumed to be from the cytosolic pool. Taking a cytoplasmic volume for $0.5x10^6$ cells of 0.58µl (cytoplasmic volume representing 75% of the total cell volume, Friedman *et al.*, 1985) the concentration of catecholamines in the cytoplasm was calculated to be approximately 2.6mM. The cytosolic sodium concentration is unknown but is thought to be in the order of 1 to 5mM (Haigh and Phillips, 1989). Therefore, in the absence of extracellular Na^+ there would be an outwardly directed catecholamine and Na^+ gradient. The results presented here, however, suggest that efflux does not occur. It is possible that the conditions imposed on the cells are not sufficient to cause efflux. This seems unlikely from the observations of efflux under essentially identical conditions in PC12 cells (Bönisch *et al.*, 1984). Alternatively, it may be postulated that the uptake carrier is in fact unidirectional and is "gated" in some way so that a mechanism of carrier-mediated efflux is not favoured.

A recent report by Powis *et al.* (1989) has also suggested that carrier-reversal is not a general mechanism of catecholamine release in chromaffin cells. Conditions of reduced extracellular Na^+ and Ca^{2+} did not produce an efflux of [^{3}H] noradrenaline within 60min. There was an efflux in the presence of Ca^{2+} but this was not inhibited by cocaine so is unlikely to have been the result of carrier-reversal. It was also found in the present study that, even in the presence of Ca^{2+}, exposure of chromaffin cells to Na^+-free conditions for up to 6h produced no release of catecholamines above basal levels (results not shown). Under the experimental conditions used, however, reversal of the plasma membrane Na^+ gradient alone would not be sufficient to mediate efflux since it has been shown in other systems (Schömig *et al.*, 1988; and Bönisch *et al.*, 1984) that both this and elevated cytosolic catecholamines are required for carrier-reversal to occur. Incubation of chromaffin cells with radiolabelled ascorbic acid results in a rapid uptake into the cells, this "newly-acquired" (NA)-ascorbate being initially found in the cytosol (Knoth *et al.*, 1987). NA-ascorbate, but not endogenous catecholamine, was released from digitonin permeabilised cells in the absence of Ca^{2+}. NA-ascorbate release evoked by veratridine was also dependent on extracellular Na^+. It is suggested that NA-ascorbate is released from the cytosol by a non-exocytotic mechanism of release; this being reversal of the Na^+-linked ascorbate symporter. In other words, some forms of carrier-reversal may occur in chromaffin cells.

The observations that the catecholamine uptake carrier does not appear to be reversible, and the well-known inability of tyramine to displace endogenous catecholamines suggest that the chromaffin cell system may be more dissimilar to adrenergic neurons than previously suspected. PC12 cells possess a tyramine releasable pool of catecholamines (Youdim *et al.*, 1986) and also demonstrate carrier-mediated efflux (Bönisch *et al.*, 1984). Furthermore, both PC12 cells and adrenergic nerve endings possess MAO type A, in contrast to MAO type B found in chromaffin cells (Youdim *et al.*, 1984). The lack of tyramine effect in chromaffin cells was suggested to be due either to lack of a tyramine accessible pool of catecholamines or absence of a specific site on the plasma membrane for release (Youdim *et al.*, 1986). When cells were permeabilised with digitonin following 4h treatment with tyramine there was no release of catecholamines above that released from untreated cells (results not shown). This suggests that chromaffin cells do not possess a catecholamine pool that is available to tyramine. In conclusion, catecholamine release caused by metabolic inhibition in chromaffin cells may be mediated by a Na^+-dependent mobilisation of internal Ca^{2+} stores. Intracellular acidosis may activate the Na^+/H^+ exchanger leading to a Na^+ influx through this antiporter prior to catecholamine secretion. A mechanism of carrier-mediated efflux which has been proposed to occur under similar conditions in cardiac sympathetic neurons does not occur in chromaffin cells, possibly due to the uptake carrier being "gated" in some way. These observations raise questions concerning the suitability of chromaffin cells as a model for adrenergic neurons.

Chapter Six

Measurement of Granule Proteins and ATP as Markers for Exocytosis

6.1 Introduction

When granules fuse with the plasma membrane during exocytosis they release not only catecholamines but also ATP and a variety of soluble proteins including DBH, chromogranins and a range of peptides. The co-release of these granule components, but not cytosolic proteins, with catecholamines has been used to distinguish exocytosis from other mechanisms of catecholamine release such as passive diffusion or carrier-mediated efflux (Kirshner and Kirshner, 1971). It has been demonstrated using specific antibodies directed against chromogranin A that this is co-released with catecholamines in the same ratio as that present in intact vesicles upon nicotinic stimulation (Kirshner and Kirshner, 1971). Measurement of granule proteins released from chromaffin cells treated with metabolic inhibitors would thus enable identification of the mode of catecholamine release, the co-release of proteins and catecholamines being indicative of a mechanism of exocytosis. In conjunction, measurement of the cytosolic protein lactate dehydrogenase can be used to distinguish exocytotic release from non-specific cell lysis.

The co-release of ATP with catecholamines has also been demonstrated by Rojas *et al.* (1985) using an online detection system, monitoring the ATP-dependent bioluminescent oxidation of firefly luciferin during acetylcholine-stimulated secretion. Measurement of ATP released together with catecholamines would provide further evidence for a mechanism of exocytosis. Under conditions of metabolic inhibition ATP is rapidly depleted from the cell and nicotine-stimulated secretion is abolished (Nakanishi *et al.*, 1988). The ATP sequestered within the chromaffin granule is metabolically inert and only exchanges with difficulty with the cytoplasmic pool (Corcoran *et al.*, 1986). Morita *et al.* (1988) demonstrated that it is the labile cytoplasmic pool of ATP that is involved in exocytosis. Digitonin permeabilisation was used to deplete the cytoplasmic pool of ATP and this led to loss of the calcium-stimulated exocytotic response. This response was restored by the addition of exogenous ATP, thus demonstrating the dependence of exocytosis on cytoplasmic ATP. By combining digitonin permeabilisation with the online bioluminescent detection of ATP it was anticipated that cytoplasmic ATP could be measured and its depletion with metabolic inhibitors demonstrated.

6.2 Results

6.2.1 Detection of granule proteins.

Two methods were used to determine the presence of, and to quantitate the

co-release of granule proteins with catecholamines. The first method used an ELISA system. A range of dilutions of granule lysate of known protein concentration were probed with specific antibodies to either DBH or chromogranin A, as described in Chapter 2. Following development of the ELISA plates the amount of antigen present in each well could be quantified by measuring the absorbance of each well at 410nm. These results are shown in Fig 6.1. There was a linear relationship between absorbance and the logarithm of the protein concentration when lysate was probed with anti-DBH within the range of 0.1μg to 1mg/ml of total lysate protein. Below this concentration the assay system was not sensitive enough to distinguish between wells of differing protein levels.

When probed with anti-chromogranin A the plot of protein concentration versus absorbance was bell-shaped. This suggested there was an optimal range over which chromogranin A can be detected using this system. At higher concentrations there was a fall in absorbance. At high protein concentrations the protein may leach off the wells and bind the antibody in suspension, the complex then being washed off the plates. Consequently a much lower signal is obtained that does not reflect the original protein concentration in the well. At concentrations below the optimum there is a decrease in absorbance with decreasing protein concentration, as is seen with detection of DBH, within the sensitivity limits of the assay.

Cell supernatants from cells treated for 10min with Locke's buffer (control), 10μM nicotine or metabolic inhibitors (M.I.) were serially diluted, following centrifugation to remove any cell debris, applied to ELISA plates and probed with either anti-DBH or anti-chromogranin A. Fig 6.2A illustrates the results plotted as log dilution factor versus absorbance at 410nm. It was not possible to distinguish between the three samples when probed with DBH. The dilution range used was quite broad so the difference in levels of DBH in the three samples may not be great enough to be able to detect any variation in absorbance.

The supernatants targeted with chromogranin A gave a similar bell-shaped graph to that seen with granule lysate (Fig 6.2B). Within what appears to be the optimal protein concentration range both the 'nicotine' and 'metabolic inhibition' supernatants contained significantly higher levels of chromogranin A than the control supernatants as indicated by the higher absorbance values ($p < 0.001$ for dilutions of 50-fold and 100-fold). The 'nicotine' supernatant possessed the highest level of chromogranin A as predicted from the catecholamine release measured in the undiluted supernatants (12% catecholamine release from

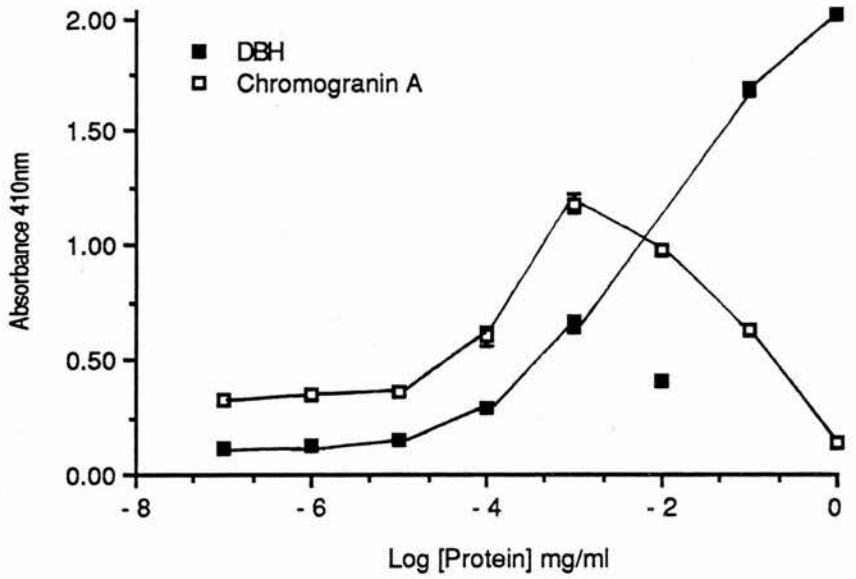


Fig 6.1 Detection of DBH and chromogranin A in granule lysate using an ELISA system. Wells were coated using 0.1ml of diluted lysate of whole chromaffin granules for 90min at 37°C. Absorbance is plotted as a function of the log of the protein concentration used during coating. Antibodies were used as described in Chapter 2. Results are expressed as mean absorbance at 410nm ± SE of 4 determinations.

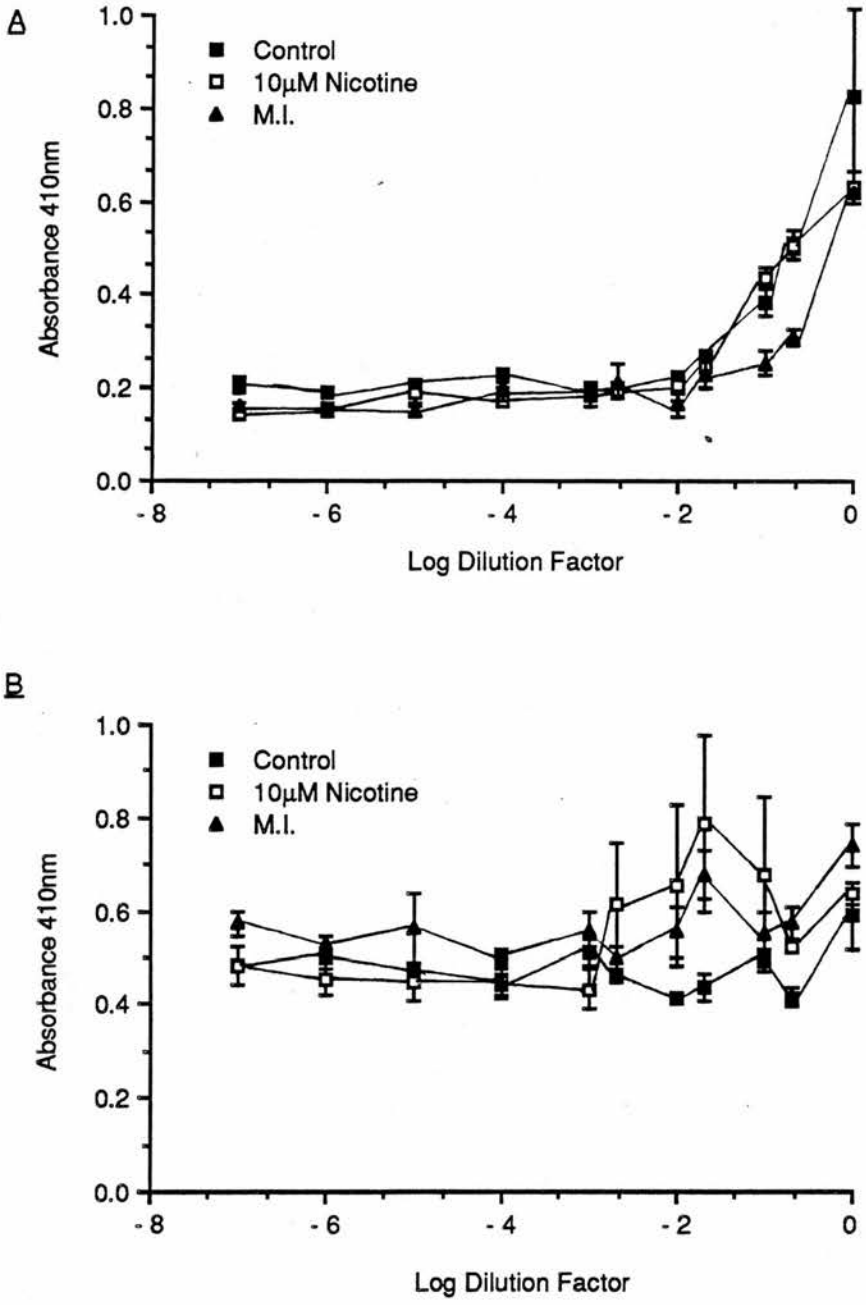


Fig 6.2 Detection of DBH in cell supernatants by ELISA. Cells were treated for 10min with Locke's buffer plus or minus 10μM nicotine or metabolic inhibitors. A range of dilutions were probed for DBH (A) or chromogranin A (B). Results are plotted as mean absorbance, 410nm ± SE of 4 determinations versus log dilution factor.

nicotine stimulated cells compared with 10.3% from cells treated with metabolic inhibitors, and 2.9% basal release).

This result suggests that under both conditions described chromogranin A is co-released with catecholamines, indicative of a mechanism of exocytosis. These results are, however, only qualitative, not quantitative, due to the lack of a simple correlation between protein content and signal, the latter being disproportionately lower at higher protein concentrations. Without a quantitative measurement of protein in the supernatant it was not possible to determine whether the catecholamine/protein ratio reflected that in the granules.

In order to quantitate the amount of chromogranin A being released under conditions of metabolic inhibition compared with nicotinic stimulation PAGE and Western blotting was utilised. It was assumed that all the protein entered the gel and the majority transferred to nitrocellulose (as indicated by staining the residual gel following transfer). In case transfer was not uniform across the nitrocellulose/gel sandwich each cell supernatant was applied several times across the full width of the gel. Granule lysate of known protein content was treated identically. Blots were then developed with antiserum, biotin-labelled second antibody and [^{125}I] streptavidin, and autoradiographed. The two gels were run, blotted and labelled in parallel so that it was possible to make a direct comparison of band intensities between the two resulting autoradiographs.

Chromogranin A was targeted in preference to DBH since the former is sequestered solely within the granule matrix. DBH is found both in the granule matrix and bound to the granule membrane. Following exocytosis membrane-bound DBH is exposed at the cell surface (Hunter and Phillips, 1989) so possible contamination of the samples with cell membrane debris could lead to a false positive result.

An approximation of the amount of protein derived from the interior of the secretory granules and present in the culture supernatants was calculated, using the known percentage of cellular catecholamine that was released in the experiment. This was then used as a guide in order to obtain a standard curve relating protein content to the intensity of the chromogranin band on the autoradiographs.

The mean catecholamine content of the three culture supernatants was 4.7 nmol/ml (Table 6.1). Using a value of 2.5μmol catecholamine/mg protein for the soluble content of a granule (Winkler and Westhead, 1980), the mean protein content of the culture supernatants was reckoned to be 1.9μg/ml. Of this, about

80% should be chromogranin A. Therefore dilutions of granule lysate to give values around this order of magnitude (0.05 to 0.4μg) were applied to separate tracks on the gel in order to construct the standard curve (of band intensity versus protein content).

The resultant autoradiograph (Fig 6.3A) and the plot of protein concentration versus peak integral (Fig 6.3B) showed a direct, linear relationship between protein concentration and band intensity, as measured using densitometry. Fig 6.4 illustrates the parallel autoradiograph obtained from the culture supernatants of treated cells, probed with the same anti-chromogranin A and ^{125}I streptavidin. Band intensities of 'nicotine' and 'metabolic inhibition' samples were significantly higher than those of the control samples. From the integrals of the densitometric peaks for each band the concentration of total soluble granule was calculated using the standard curve (Fig 6.3B). These results are shown in Table 6.1.

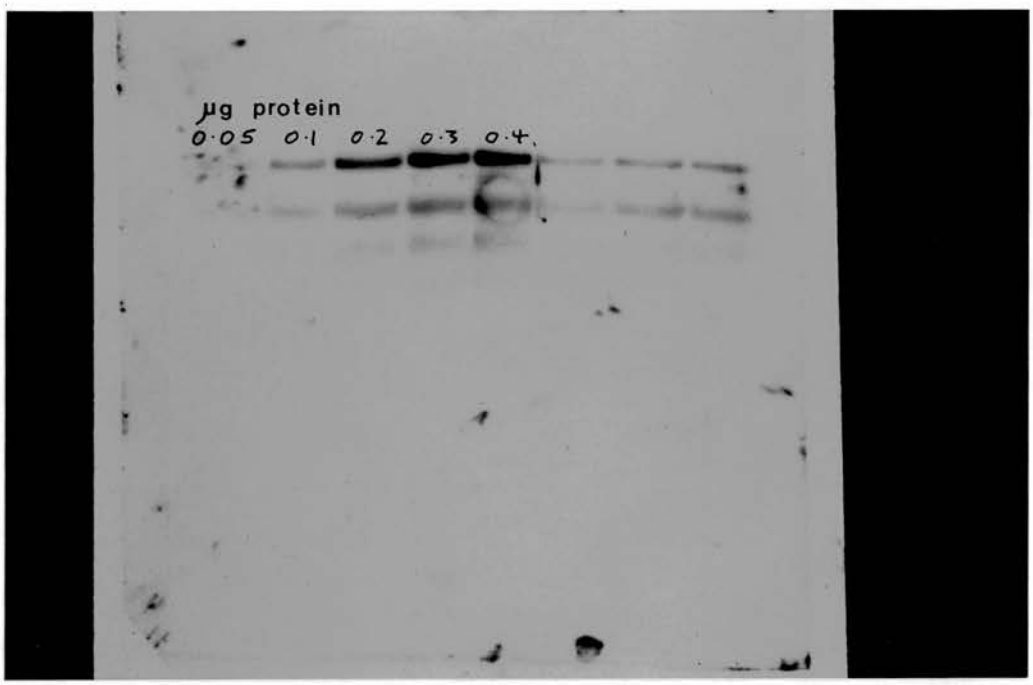
Table 6.1

Content of soluble granule proteins in culture supernatants under conditions of nicotine stimulation or metabolic inhibition. Catecholamine release is measured as percentage of total catecholamine in the cells, and protein was calculated from the standard curve (Fig 6.3) and is presented in μg/ml of culture supernatant (mean ± SE of three determinations).

Sample	Catecholamine release (%)	Catecholamine in supernatant nmol/ml	Soluble granule protein in supernatant μg/ml	Ratio CA μmol/mg protein
Control	4.1 ± 0.18	2.35	1.56 ± 0.02	1.51
10μM Nicotine	12.9 ± 0.22	7.58	3.88 ± 0.05	1.95
Metabolic Inhibition	9.1 ± 0.23	5.13	3.25 ± 0.03	1.58

The amount of protein released from the stimulated cells was significantly higher than that released under basal conditions ($p<0.001$). Taking a value of 2.5μmol catecholamine/mg protein in the granules it was predicted that if granule proteins were co-released with catecholamines values of 0.95μg (control); 3.0μg (10μM nicotine); and 2.05μg (metabolic inhibition) were expected. The actual values obtained (Table 6.1) were all higher than the predicted values. The catecholamine (CA)/protein ratio was between 1.5 and 2μmol/mg protein (Table 6.1).

A



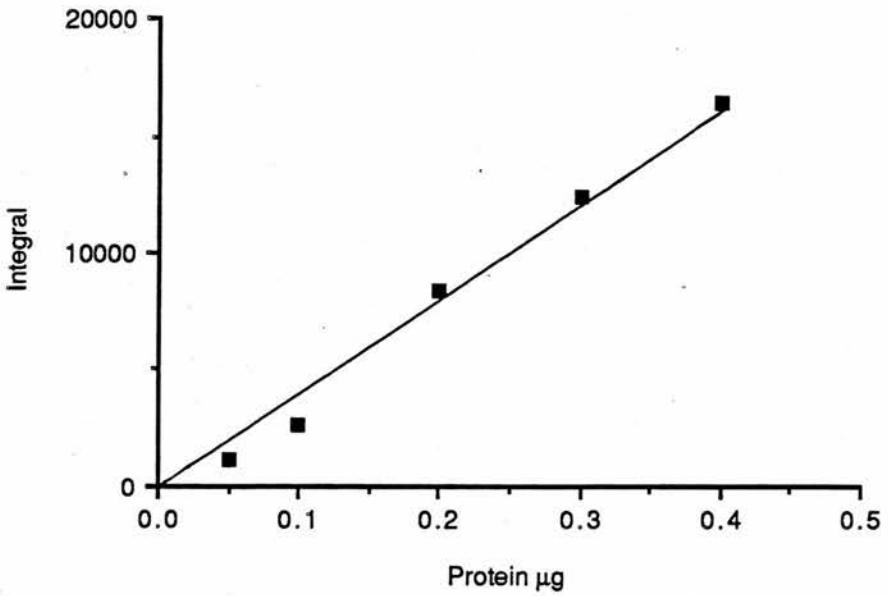


Fig 6.3 A. Autoradiograph of chromogranin A detected in granule lysate over a protein range of 0.05 to 0.4µg. Chromogranin A was detected using anti-chromogranin A and IgG-biotin linked to ^{125}I-streptavidin following PAGE and Western blotting.
B. Plot of band intensities measured by densitometry (integral of peak, arbitrary units) versus total protein loaded on gel track (µg).

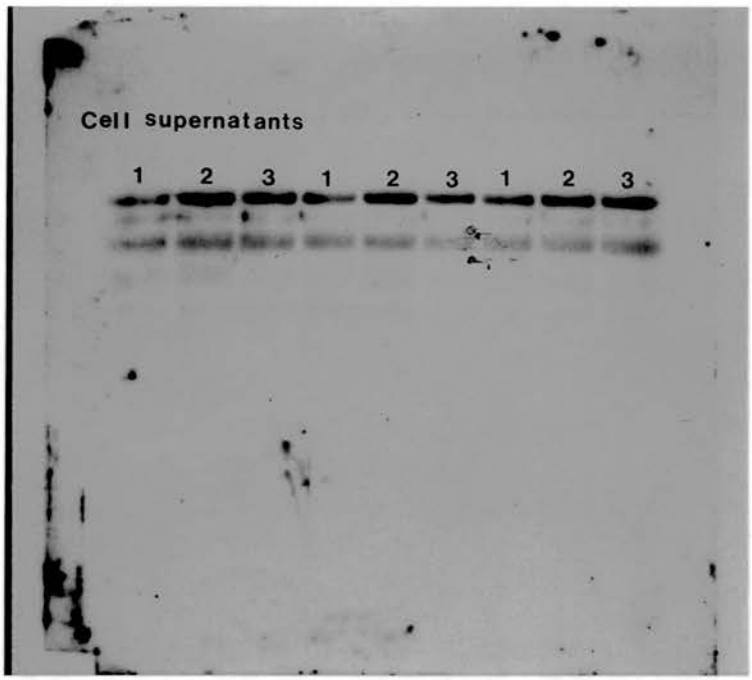


Fig 6.4 Autoradiograph of chromogranin A detected in culture supernatants from cells treated with Locke's buffer (tracks 1), 10μM nicotine (tracks 2), or metabolic inhibitors (tracks 3).

The catecholamine content of granules is approximately 2.5μmol/mg total protein (Winkler and Westhead, 1980). As only 80% of this is soluble protein the catecholamine/soluble protein ratio is probably closer to 3μmol/mg protein. There will, however, be deviations from this value as the relative amounts of catecholamines and protein varies in different cell populations, and also noradrenaline containing granules may differ from adrenaline containing granules. In addition, catecholamines may be taken up again or metabolised. The evidence presented here is strongly suggestive of chromogranin A being co-released with catecholamines under conditions of both metabolic inhibition and nicotine stimulation.

This was backed up by measurement of neuropeptide Y by radioimmunoassay (Richardt *et al.*, 1988) which is also sequestered within the secretory granules. These results are presented in Table 6.2.

Table 6.2

Detection of neuropeptide Y in culture supernatants under conditions of nicotine-stimulation or metabolic inhibition.

Sample	Catecholamine release nmol/ml	Neuropeptide Y nmol/ml
Control	2.68 (3.1%)	0.07 ± 0.008 (8)
10μM Nicotine	5.18 (12.0%)	0.19 ± 0.02 (9)
Metabolic Inhibition	4.42 (10.3%)	0.23 ± 0.01 (6)

Catecholamine release is presented in nmol/ml with percentage release in parenthesis. Neuropeptide Y is presented in nmol/ml ± SE (number of determinations in parenthesis).

There was a significant increase in the release of neuropeptide Y following stimulation with either nicotine or metabolic inhibitors ($p<0.001$). It was not possible, however, to make a direct comparison of neuropeptide Y with catecholamine release as even in the control samples the amount of neuropeptide Y present was at the upper limit of the detection system. The 'nicotine' and 'metabolic inhibition' samples may have been too concentrated for the radioimmunoassay and so the values obtained are probably underestimates of the amount of neuropeptide Y present. This might account for the higher neuropeptide

Y concentration in the 'metabolic inhibition' samples despite the percentage catecholamine release being lower than that obtained with nicotinic stimulation.

6.2.2 Detection of the cytosolic protein, lactate dehydrogenase.

The results presented above suggest that both chromogranin A and neuropeptide Y are co-released with catecholamines under conditions of metabolic inhibition. This is indicative of a mechanism of exocytosis. It is plausible, however, that these conditions could cause non-specific cell lysis although from evidence discussed in Chapter 4 this would appear unlikely. To confirm that the cells maintain their integrity under conditions of metabolic inhibition lactate dehydrogenase was assayed in parallel with catecholamines. There was no release of lactate dehydrogenase above basal levels under conditions of either metabolic inhibition or nicotine stimulation (Fig 6.5). In contrast, digitonin permeabilisation which renders the plasma membrane leaky caused 18% of the total cellular lactate dehydrogenase to be released over 10min. There was only 0.9% catecholamine release, under these conditions, indicating that the granules remain intact. In conclusion, therefore, under the conditions described, the cells maintain their integrity and catecholamines are released by a mechanism of exocytosis.

The method of digitonin permeabilisation was used in a further confirmation of this. Leakage of catecholamines from the granules into the cytosol under conditions of metabolic inhibition should result in an increase in catecholamines released by permeabilising the cells with digitonin. Cells exposed to 10μM digitonin following 10min metabolic inhibition showed no elevation of catecholamine release compared to release from control cells, even in the presence of pargyline (results not shown), further confirming that release by the action of metabolic inhibitors is exocytotic in origin.

6.2.3 Bioluminescent detection of ATP.

ATP released during exocytosis can be directly and continuously detected by the luciferin-luciferase assay method. As shown in Fig 2.2 the light response was logarithmically related to the concentration of ATP present.

Initial assays using chromaffin cells in suspension were carried out on approximately 10^4 cells. It was found, however, that the ATP content measured following cell lysis with Triton X-100 was consistently about 100 times lower than was predicted from the catecholamine content of the cells. This could be due to the presence of some factor in the cell suspension inhibiting the luciferin-luciferase assay system or alternatively due to rapid hydrolysis of ATP

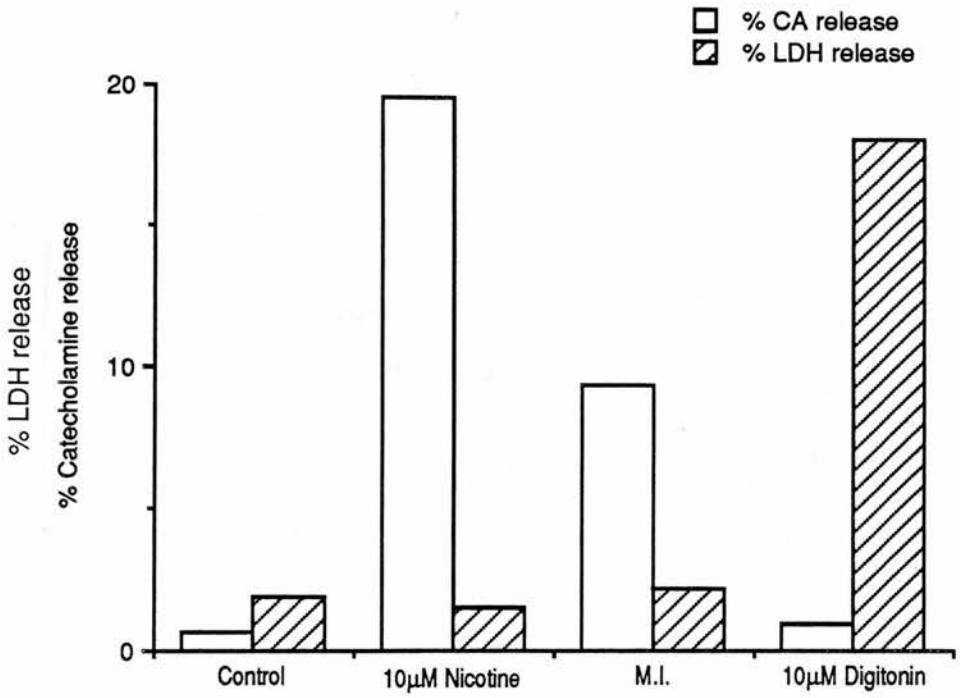


Fig 6.5 Measurement of lactate dehydrogenase. Cells were treated for 10min with Locke's buffer (control), 10μM nicotine, metabolic inhibitors (M.I.), or 10μM digitonin (in calcium-free buffer). Catecholamine and LDH were measured in culture supernatants and residual amounts measured following lysis with 1% Triton X-100. Results are expressed as percent release, means ± SE of triplicate determinations.

by ATPases which are released on cell lysis. Therefore, following cell lysis internal standards of known ATP concentration were included in the assay system. Exogenous ATP was added at a concentration similar to that found in the cells ($5x10^5$ cells contain 50nmol catecholamine which is equivalent to 11nmol ATP, assuming a catecholamine/ATP ratio of 4.5) and the light intensity monitored for 20min. Over this time there was no reduction in the light intensity and this was the same as that obtained in the absence of cells, indicating that the assay system is not affected by the presence of cells or by the detergent Triton X-100. In addition, over this time, loss of ATP by hydrolysis does not seem to be a problem. Inclusion of an ATP regenerating system (1μmol phosphoenolpyruvate and 5mU pyruvate kinase) did not affect the light intensity of exogenous ATP in the presence of cells. Thus the discrepancy between the amount of ATP measured by this assay method and the predicted amount could not be accounted for.

One possibility was that ATP released into the immediate cell surroundings was hydrolysed by local ATPases which did not come into contact with exogenous ATP. Alternatively there may be some factor co-released from the granules with ATP that affected its detection. For instance, within chromaffin granules ATP is in association with catecholamines and possibly also matrix proteins (Koppell and Westhead, 1982). This could reduce the amount of ATP available to the assay system, which would not be seen with exogenous ATP. Therefore the ATP content of lysed granules was measured over a range of dilutions and the catecholamine content measured in parallel.

Granule lysate was diluted with assay buffer over a 10,000 fold range and samples assayed in triplicate for ATP and catecholamine content. Fig 6.6 shows the relationship between ATP and catecholamine contents. There was a good correlation ($r=0.98$) between the two variables. From the regression line a molar ratio of 8.6 catecholamine/ATP was calculated. Winkler and Westhead (1980) reported a molar catecholamine/ATP ratio of 4.5. However ratios of 8.2,10 and 12 have also been reported (for discussion see Winkler and Westhead, 1980). Chromaffin granules appear to be non-homogeneous in terms of catecholamine and ATP content so the ratio obtained here is not necessarily inconsistent with other observations. In any case, it can be concluded that the ATP assay is not affected by any granule components and ATP in the matrix can be assayed by this method.

Rojas *et al.* (1986) reported that emitted light was linearly related to ATP levels in chromaffin cells, provided the concentration of cells in the reaction mixture

was kept below 10^5 cells/ml. In their experiments they generally used less than $2.5x10^5$ cells/ml in order to minimise ATP hydrolysis by ecto-ATPases. I therefore used approximately 10,000 cells in 200µl samples (i.e. $0.5x10^5$ cells/ml), assaying their ATP content following lysis with Triton X-100. Under these conditions the amount of ATP measured was close to that predicted from the catecholamine content (assuming a catecholamine/ATP ratio of 4.5). Values ranging from 15 to 40nmol/10^6 cells were calculated. Taking an average catecholamine content of 100nmol/10^6 cells (Phillips, 1977) the ATP content was predicted to be about 22nmol/10^6 cells. The values found were generally within the predicted range for these very dilute cell suspensions, taking into consideration the variability in catecholamine content of different cell preparations and consequent likely variations in ATP content, and also the fact that the number of cells in each assay was only known approximately.

The release of ATP in the presence or absence of different secretagogues was monitored over 10min. Readings were taken every min and a time course of secretion plotted (Fig 6.7). After 10min a known amount of ATP was added to check the calibration of the system. The signals from exogenous ATP did not significantly deviate from the signal obtained with the same ATP concentration in the absence of cells. Alternatively at the end of each time course cells were lysed with Triton X-100 to determine the total cell ATP content. After 10min the % total ATP released was 2.5% ± 0.8 (control); 8.3% ± 1.0 (10µM nicotine); and 6.1% ± 1.6 (metabolic inhibition). These values are similar to those reported by Rojas et al. (1985), 10µM acetylcholine releasing approximately 8% of total ATP in 2min in their experiments. The time course of secretion was consistent with that obtained for catecholamine secretion (Figs 3.6 and 4.4).

Nicotine-stimulation gave the greatest response, the bulk of secretion occurring within 2min of the addition of the agonist. In contrast, the release evoked by metabolic inhibitors was smaller and the response slower, a gradual increase in ATP being seen with time. Therefore under conditions of both cholinergic stimulation and metabolic inhibition ATP release seems to parallel catecholamine secretion, suggesting that both are released from the same cellular pool.

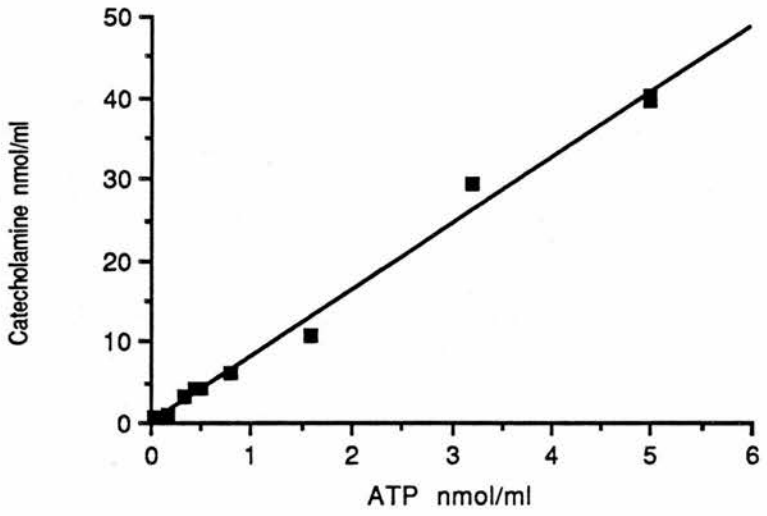


Fig 6.6 Plot of catecholamine nmol/ml versus ATP nmol/ml measured in granule lysate. Results are the means of triplicate determinations (error bars omitted for clarity) and the best-fit line drawn by regression analysis.

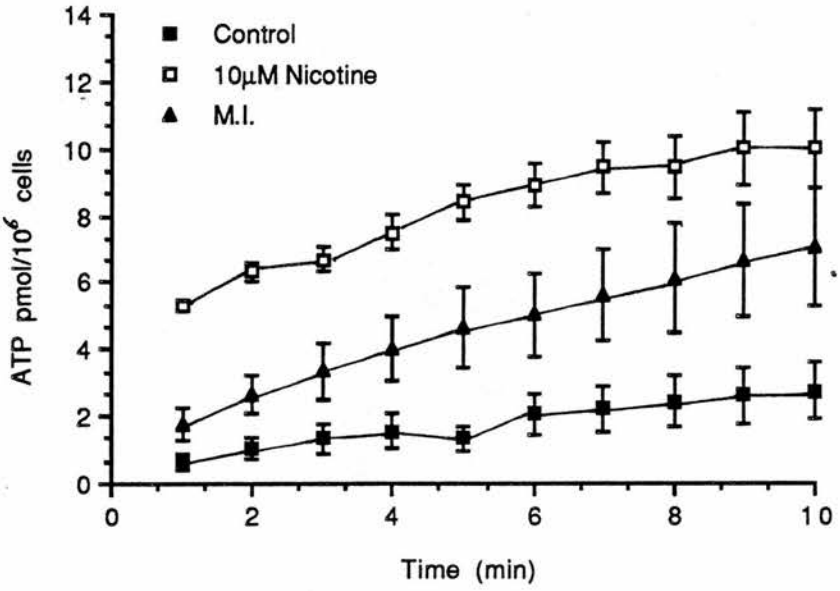


Fig 6.7 Time course of ATP release over 10min from 10^4 cells in 200μl treated with Locke's buffer (control), 10μM nicotine or metabolic inhibitors (M.I.). Results are expressed as pmol ATP/10^6 cells, means ± SE of triplicate determinations.

6.3 Discussion

6.3.1 Exocytotic release.

Immunological techniques have been used to demonstrate an exocytotic mechanism of release after stimulation of chromaffin cells with various secretagogues. Following stimulation with nicotine or metabolic inhibitors there was a significant release of chromogranin A from the cells compared with basal release. The catecholamine/chromogranin A ratio in the supernatant was similar to that in intact granules, indicating that both are released from the same cellular store. Elevated neuropeptide Y was also found following stimulation of cells. It was not possible, however, to obtain a reliable quantitative estimate of the molar ratio of neuropeptide Y to catecholamine. Neuropeptide Y is known to be sequestered within the chromaffin granule along with catecholamines and other soluble proteins (Winkler and Westhead, 1982).

The co-release of neuropeptide Y has also been reported in isolated perfused guinea pig hearts following nicotine or electrical stimulation (Richardt *et al.*, 1988; and Haass *et al.*, 1989). Release of both catecholamines and neuropeptide Y were Ca^{2+} sensitive and modulated by presynaptic regulation. Non-exocytotic release of noradrenaline induced by tyramine perfusion of hearts did not evoke co-release of neuropeptide Y. Similarly anoxia evoked a noradrenaline overflow which was sensitive to desipramine, but there was no concomitant release of neuropeptide Y (Haass *et al.*, unpublished observations). This absence of neuropeptide Y was taken as a further indication of carrier-mediated efflux of noradrenaline during anoxia.

Sympathetic neurons contain both large and small dense core vesicles (Winkler, 1988). The large dense core vesicles contain catecholamines, ATP, chromogranins, enkephalins and neuropeptide Y. The small dense core vesicles are known to contain noradrenaline and ATP, but do not contain chromogranin A, enkephalins or neuropeptide Y. It might be postulated that under conditions of anoxia the sensitivity of the two different types of vesicles to the metabolic changes is altered so that only the small dense core vesicles release their contents to the exterior, accounting for the absence of neuropeptide Y. Furthermore small dense core vesicles located at the nerve terminal seem to go through several cycles of release. Under anoxic conditions they may be more readily able to release their contents, even in the absence of ATP, being close to the site of release, unlike large dense core vesicles. The latter are formed in the cell body and transported to the

nerve terminal by axoplasmic flow. In the absence of ATP the processes required for transport of the large dense core vesicles to the sites of release and subsequent exocytosis would be inhibited.

Chromaffin cells possess only one type of secretory vesicle. In these cells the co-release of chromogranin A, neuropeptide Y and catecholamines under conditions of metabolic inhibition suggests that release was exocytotic, the absence of lactate dehydrogenase in the supernatant indicating that the cells maintained their integrity.

Exocytotic secretion from electrically permeabilised chromaffin cells has an absolute requirement for exogenous Mg-ATP (Baker and Knight, 1981). Additionally, conditions of metabolic inhibition cause a rapid reduction in cytoplasmic ATP and catecholamine secretion evoked by carbamylcholine in intact chromaffin cells and by direct calcium challenge in digitonin permeabilised cells (Nakanishi *et al.*, 1988). In contrast, Reynolds *et al.* (1982) reported that metabolic inhibitor-induced depletion of cellular ATP in PC12 cells did not result in any inhibition of acetylcholine or dopamine release evoked by carbachol or high K^+.

Recently Holz *et al.* (1989) reported that in the absence of Mg^{2+} and ATP both intact and permeabilised chromaffin cells secrete a small, but significant amount of catecholamine in response to elevated Ca^{2+}. It is suggested that intact cells are primed by intracellular ATP so that upon permeabilisation there is an element of release that is ATP-independent. Furthermore, if granules are adjacent to the plasma membrane, ATP may not be required and exocytosis could occur spontaneously under certain conditions, such as metabolic inhibition.

6.3.2 ATP measurements

By monitoring the bioluminescent oxidation of luciferin it should be possible to demonstrate a rundown of cytoplasmic ATP in the presence of metabolic inhibitors. The cytoplasmic pool of ATP represents only 10% of the ATP in the cell. Therefore, initial experiments concentrated on measuring granule ATP released following nicotine stimulation, and total cell ATP liberated by detergent in order to establish a suitable method of detection. However, it became apparent that this assay method had severe limitations. Both the enzyme system and ATP are highly labile which necessitated repeated calibration. In addition, when a scintillation counter is used 'in-coincidence' as a photon counter it is necessary for two pulses, one from each photomultiplier, to be detected within $3x10^{-8}$sec to

record a signal. This limits the sensitivity of the assay since the number of signals is much lower than the number of photons produced at very low ATP concentrations. At this lower end of the standard curve background noise also masks the real signal. At high ATP concentrations more than a single pair of photons are produced at any one time and the detection system is not sensitive enough to discriminate between them, the reading obtained being an underestimate of the light produced (as discussed by Stanley and Williams, 1969).

Additionally it was found that with an increasing number of cells in the assay (up to $5x10^5$/ml) the signal was approximately 100 times lower than that expected. Only when less than 10^5 cells/ml were assayed did the amount of ATP correlate with the catecholamine release. The reason for this apparent discrepancy is not very clear. This has previously been reported by Rojas *et al.* (1985), being attributed in that case to ecto-ATPases.

It has, however, been possible to demonstrate the co-release of catecholamines and ATP following treatment of cells with either nicotine or metabolic inhibitors; the extent of ATP release being greater than basal release and of a similar magnitude and time course to catecholamine release.

The cytosolic ATP pool, which represents only approximately 10% of the total cellular ATP, has been measured following digitonin permeabilisation (Rojas *et al.*, 1985). Treatment of cells with metabolic inhibitors specifically depletes this cytoplasmic pool and secretion after 30min in the presence of dinitrophenol (Nakanishi *et al.*, 1988). Under the conditions described here it did not prove possible to make a direct measurement of the cytoplasmic ATP pool or to demonstrate its depletion with metabolic inhibitors. There is, however, indirect evidence: following 10min treatment with metabolic inhibitors, the response to nicotine stimulation or to high K^+ is completely abolished. Uptake of [^{3}H] noradrenaline is also inhibited in the presence of metabolic inhibitors. These observations provide indirect evidence for the depletion of the cytoplasmic ATP pool as it is this pool that is metabolically active. Thus this exocytotic release is apparently independent of cytoplasmic ATP.

Chapter Seven

Role of $[Ca^{2+}]_i$ in Catecholamine Release Induced by Metabolic Inhibition

7.1 Introduction

It is now widely recognised that exocytosis in the chromaffin cell has an absolute requirement for extracellular Ca^{2+}. Following stimulation with a nicotinic agonist or direct depolarisation with high K^+, the influx of Ca^{2+} through voltage-dependent Ca^{2+} channels raises cytosolic Ca^{2+} and triggers a chain of events that leads ultimately to exocytosis.

The rise in $[Ca^{2+}]_i$ is transient, returning to basal levels within minutes (as described in cell populations loaded with quin-2, Knight and Kesteven, 1983; and in single cells loaded with aequorin, Cobbold *et al.*, 1987). There are probably several mechanisms for maintaining resting Ca^{2+} levels and for removing Ca^{2+} from the cell following exocytosis. Intracellular organelles such as mitochondria, endoplasmic reticulum and chromaffin granules are potential storage sites for calcium. The chromaffin granules may also play a role in removal of Ca^{2+} from the cell via exocytosis, along with the Ca^{2+} pump and Na^+/ Ca^{2+} exchanger of the plasma membrane itself.

The role of intracellular organelles in calcium homeostasis was investigated in permeabilised cells by Kao (1988). Following stimulation with acetylcholine it was found that only 5 to 10% of the resultant calcium influx was subsequently sequestered by intracellular organelles. Most of this was taken into the endoplasmic reticulum which is thought to be responsible for buffering small fluctuations in resting calcium levels. The chromaffin granules and mitochondria did not appear to have an important role. Consequently, following a rise in $[Ca^{2+}]_i$ most must be removed from the cell across the plasma membrane via the Ca^{2+} pump and the Na^+/Ca^{2+} exchanger. Exocytotic release of calcium from chromaffin granules has been demonstrated (von Grafenstein and Powis, 1989), but this is thought to be of relevance mainly to long term Ca^{2+} homeostasis rather than to the immediate restoration of resting Ca^{2+} levels following stimulation and Ca^{2+} influx.

The use of Ca^{2+}-sensitive fluorescent indicators and permeabilisation techniques has allowed direct access to the cytosol and visualisation of the changes in $[Ca^{2+}]_i$ in situ during the exocytotic event itself. These studies have revealed the potential involvement not only of extracellular Ca^{2+} but also of intracellular Ca^{2+} released from subcellular organelles in stimulus-secretion coupling.

Using the fluorescent indicator quin-2 Knight and Kesteven (1983) made direct

measurements of the resting $[Ca^{2+}]_i$ and found it to be approximately 100nM. It increased transiently on stimulation with acetylcholine or high K^+; this rise was complete within seconds, and decayed over minutes. An elevation of $[Ca^{2+}]_i$ to around 300 to 500nM was sufficient to trigger catecholamine release following stimulation with carbamylcholine (Burgoyne, 1984); and this rise in $[Ca^{2+}]_i$ was totally dependent on external Ca^{2+}. However, an elevation of $[Ca^{2+}]_i$ to 300nM induced by application of the Ca^{2+} ionophore A23187 was not sufficient to trigger secretion, suggesting the involvement of an additional second messenger in the nicotinic response.

Studies on single chromaffin cells microinjected with aequorin have revealed that the Ca^{2+} transient in response to nicotinic stimulation lasts only 60 to 90s. The transient is more prolonged in cell populations loaded with quin-2 due to the heterogeneity of the response within a population of cells (Cobbold *et al.*,1987). Use of the Ca^{2+}-sensitive indicator, Fura-2, has allowed monitoring of changes in $[Ca^{2+}]_i$ in single cells within the range of hundreds of milliseconds and visualisation and spatial resolution of the calcium signal using digital video imaging. This technique has revealed details of the calcium signal in response to a variety of secretagogues (O' Sullivan *et al.*, 1989; and O' Sullivan and Burgoyne, 1989).

Within 2s after stimulation with nicotine or high K^+ there was an elevation of $[Ca^{2+}]_i$ that was limited to a region just beneath the plasma membrane. This is attributed to entry of extracellular Ca^{2+} via voltage-dependent and, in the case of nicotine, receptor-linked Ca^{2+} channels. Following this there is diffusion of calcium throughout the cell and a second larger rise in Ca^{2+} internally. This latter event is due to the release of Ca^{2+} from intracellular storage sites and itself depends on the initial entry of external Ca^{2+}. Secretion occurs over the whole surface of the cell, as demonstrated in single chromaffin cells cocultured with Fura-2 loaded NIH-3T3 cells, the latter fluorescing in response to ATP which is co-released with catecholamines from the chromaffin cell (Cheek *et al.*, 1989a). The catecholamine release evoked by metabolic inhibition may be triggered by a rise in $[Ca^{2+}]_i$ mobilised from intracellular stores rather than from an influx of extracellular Ca^{2+}, since release is independent of external Ca^{2+} (see Fig 4.5). Such a response has been observed using Ca^{2+}-indicators in bovine chromaffin

cells when stimulated with muscarinic agents(Cheek and Burgoyne, 1985; O' Sullivan and Burgoyne, 1989; and Cheek *et al.*, 1989b). There is a rise in $[Ca^{2+}]_i$ that is totally independent of extracellular Ca^{2+}. A similar response is seen with both bradykinin and angiotensin II. These agonists trigger a receptor-mediated activation of phospholipase C leading to phosphoinositide breakdown and accumulation of IP_3. Generation of IP_3 triggers the release of Ca^{2+} from IP_3 sensitive stores. This elevation of $[Ca^{2+}]_i$ is limited to distinct sites within the cell, and digital imaging has shown the rise in $[Ca^{2+}]_i$ to be associated with one pole of the cell.

The muscarinic rise in $[Ca^{2+}]_i$ is of a similar magnitude to that seen with nicotine, but is not associated with catecholamine secretion. It is probable that the rise in $[Ca^{2+}]_i$ occurs at the pole of the cell occupied by the nucleus, endoplasmic reticulum and Golgi, away from the secretory granules. The IP_3-sensitive component of the endoplasmic reticulum is often found to be localised at one pole of the cell. A similar pattern is seen with bradykinin and angiotensin II, which trigger a small secretory response. In support of this, the catecholamine release in response to angiotensin II was polarised and was associated with the same pole of the cell as the rise in $[Ca^{2+}]_i$ (Cheek *et al.*, 1989b). The magnitude of the $[Ca^{2+}]_i$ elevation in response to bradykinin and angiotensin II was greater than that seen with muscarine, and this may explain their ability to evoke catecholamine secretion proportionate to the size of the $[Ca^{2+}]_i$ rise. In the case of bradykinin, stimulation was associated with a secondary influx of Ca^{2+} through the plasma membrane which may be the mediator of the secretory response, rather than the initial release of Ca^{2+} from internal stores. Catecholamine release in response to bradykinin, angiotensin II and methacholine is significantly reduced in the absence of extracellular Ca^{2+} but the rise in $[Ca^{2+}]_i$ remains unchanged (O' Sullivan and Burgoyne, 1989). Thus while these agents are able to mobilise internal calcium stores this event alone is not associated with a significant release of catecholamines. However, in other species, e.g rat and guinea pig, muscarinic stimulation is able to evoke catecholamine release from adrenal glands and this is known to occur in the absence of extracellular calcium (Nakazato *et al.*, 1988).

Ca^{2+} is also released internally from the mitochondria of hepatocytes under conditions of anoxia (Nishida *et al.*, 1989), this efflux being induced by a decrease in intramitochondrial ATP. This Ca^{2+} efflux is then associated with the subsequent loss of oxidative phosphorylation capacity and irreversible cell injury seen in ischaemic myocytes. In chromaffin cells the mitochondria are relatively metabolically inert in buffering cytosolic Ca^{2+} levels following a rise in $[Ca^{2+}]_i$ (Kao, 1988). Calcium uptake by the mitochondria in chromaffin cells only became significant when the calcium concentration was increased above micromolar levels. The catecholamine release evoked by metabolic inhibitors may be triggered by Ca^{2+} efflux from mitochondria induced by ATP rundown which occurs under these conditions. In the following experiments the calcium indicator Fura-2 was utilised to investigate the relationship between $[Ca^{2+}]_i$ and metabolic inhibition in chromaffin cells.

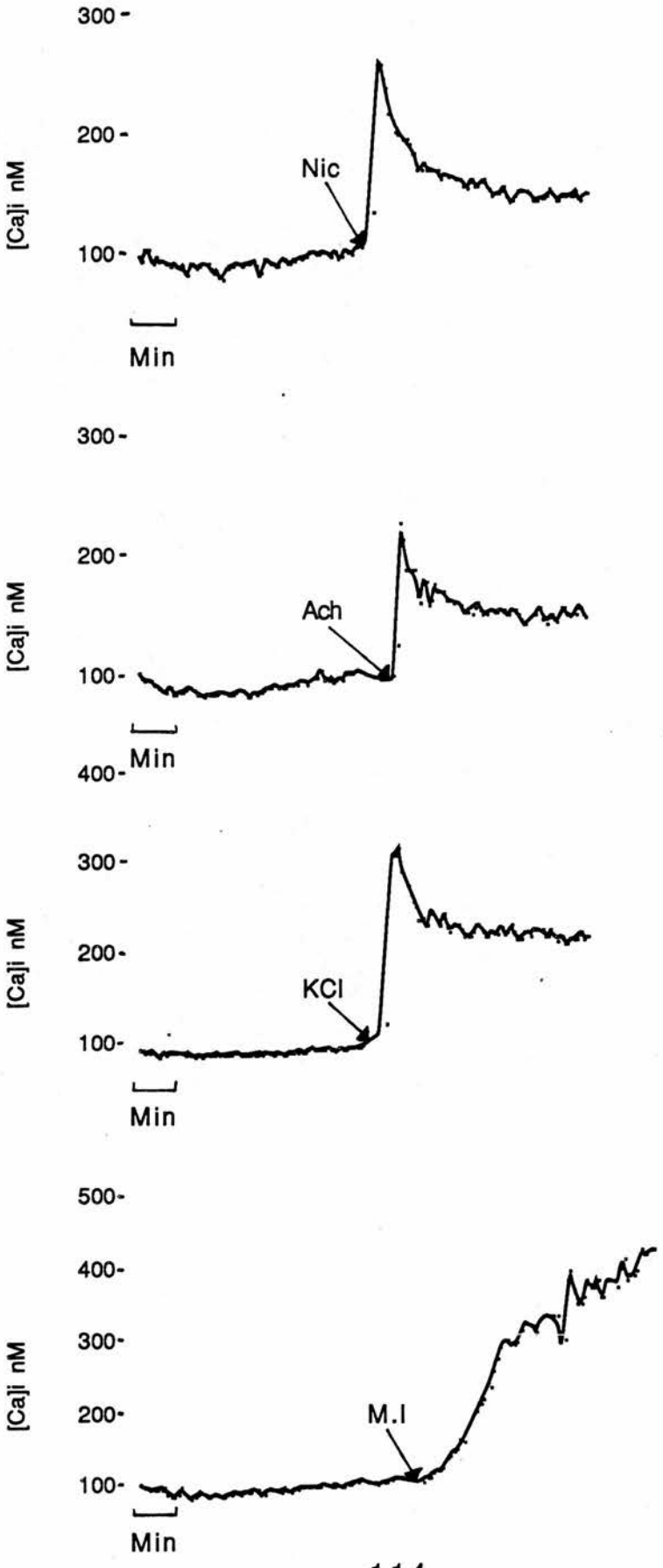
7.2 Results

7.2.1 Measurements of $[Ca^{2+}]_i$

Fig 7.1 shows the rise in $[Ca^{2+}]_i$ in chromaffin cell suspensions in response to stimulation with nicotine, acetylcholine and high K^+, and under conditions of metabolic inhibition. Cholinergic stimulation and depolarisation with high K^+ produced a rapid elevation of $[Ca^{2+}]_i$ from resting levels to a maximum of between 200 and 500nM within seconds, which then gradually fell over subsequent minutes. Similar calcium transients have previously been reported by other workers (Burgoyne and Cheek, 1985; Cheek and Burgoyne, 1985; and Cobbold *et al.*, 1987). In the absence of extracellular Ca^{2+} there was no rise in $[Ca^{2+}]_i$ in response to nicotine or high K^+ (Fig 7.2A). Upon readdition of 3mM Ca^{2+} an immediate rise in $[Ca^{2+}]_i$ was observed, again consistent with previous reports (Burgoyne and Cheek, 1985). However, $[Ca^{2+}]_i$ remained elevated for at least 2 to 3min after the readdition of Ca^{2+}. In the case of K^+ depolarisation the voltage-dependent Ca^{2+} channels remain open in the presence or absence of external Ca^{2+}, even though the secretory response has terminated (Burgoyne and Cheek, 1985). $[Ca^{2+}]_i$ also remained elevated following nicotine stimulation (Fig 7.2A) suggesting the transient nature of the secretory response cannot be solely attributed to receptor desensitisation and closure of voltage-dependent Ca^{2+}

Fig 7.1 Changes in $[Ca^{2+}]_i$ in chromaffin cell suspensions in response to nicotine, acetylcholine, high K+ and metabolic inhibitors. Basal $[Ca^{2+}]_i$ was monitored for 5min then media (all containing 3mM Ca^{2+}) were supplemented with 10μM nicotine (nic); 0.1mM acetylcholine (Ach); 50mM KCl (KCl) or metabolic inhibitors (M.I).

Fig 7.1


300
200
100
[Ca]i nM
Nic
Min
300
200
100
[Ca]i nM
Ach
Min
400
300
200
100
[Ca]i nM
KCl
Min
500
400
300
200
100
[Ca]i nM
M.I
Min

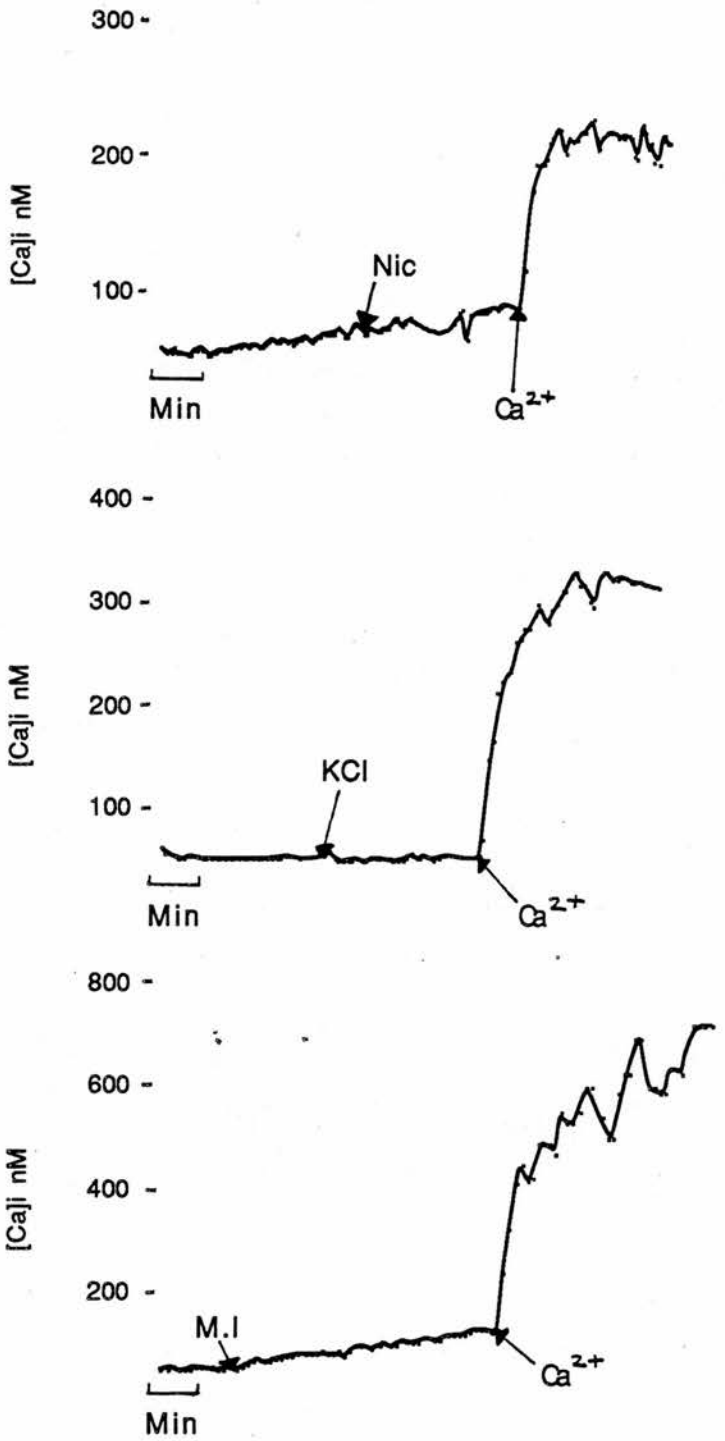


Fig 7.2A Changes in $[Ca^{2+}]i$ in chromaffin cell suspensions in response to nicotine, high K^+ and metabolic inhibitors in the presence and absence of external Ca^{2+}. The response to10μM nicotine (nic); 50mM KCl (KCl); or metabolic inhibitors (M.I) was monitored in Ca^{2+}-free KRB and upon readdition of 3mM Ca^{2+}.

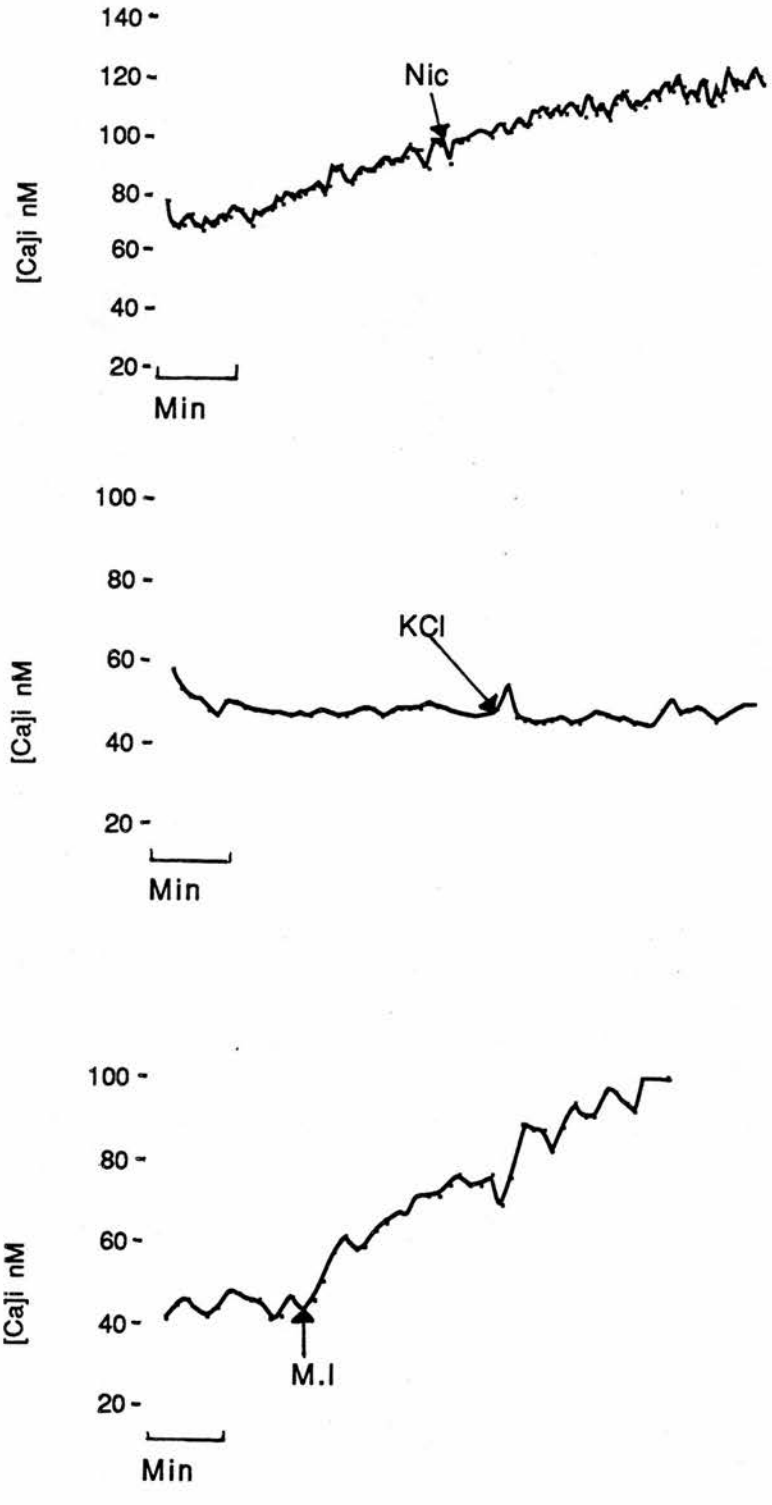


Fig 7.2B Response of cells to nicotine, high K^+ or metabolic inhibitors in the nominal absence of external Ca^{2+}. The figure is an enlargement of Fig 7.2A over the Ca^{2+}-free period.

channels (Burgoyne and Cheek, 1985).

Treatment of cells with metabolic inhibitors in the presence of extracellular Ca^{2+} produced a rise in $[Ca^{2+}]_i$ comparable in magnitude to that seen with nicotine or high K^+ (Fig 7.1). However, this elevation of $[Ca^{2+}]_i$ was slower than that seen with other secretagogues and $[Ca^{2+}]_i$ remained at this elevated level for at least 5min. This response was seen with several cell preparations, $[Ca^{2+}]_i$ rising to a plateau over several minutes.

When Ca^{2+} is omitted from the external medium metabolic inhibitors evoked a small but significant rise in $[Ca^{2+}]_i$ (Fig 7.2B). Readdition of external Ca^{2+} (Fig 7.2A) produced a large rise in $[Ca^{2+}]_i$ as previously seen (Fig 7.1). The slow and relatively small rise in $[Ca^{2+}]_i$ can only be seen in the absence of external Ca^{2+}. The latter effect may be the result of mobilisation of intracellular Ca^{2+} stores (extracellular Ca^{2+} being nominally absent). Thus there may be two elements to the rise in $[Ca^{2+}]_i$, one that results from an influx of external Ca^{2+}, and a much smaller rise which is the result of released intracellular Ca^{2+} and which is seen in the absence of external calcium.

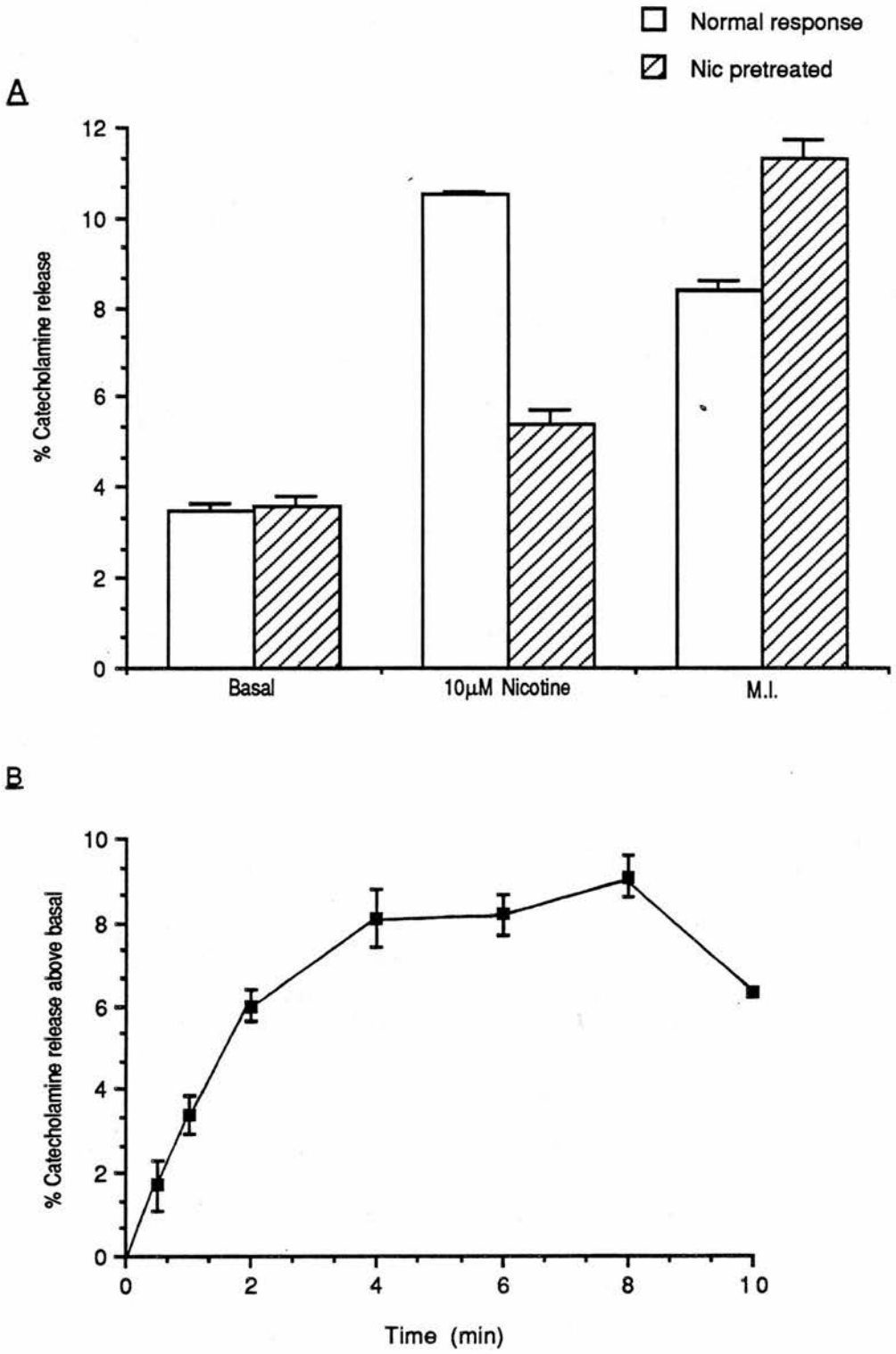
7.2.2 Effect of nicotine pretreatment

Further questions about the nature of catecholamine release evoked by metabolic inhibition were raised following observation of the effect of nicotine pretreatment. When cells were treated for 10min with 10μM nicotine and the supernatant was then replaced with buffer containing metabolic inhibitors, the subsequent catecholamine release was significantly greater than that seen with metabolic inhibitor treatment alone (Fig 7.3A). This potentiated response was not seen if the cells were pretreated for 10min with Locke's buffer alone or buffer containing 50mM K^+.

The inability of high K^+ to potentiate the response evoked by metabolic inhibitors suggested that it must be some feature of nicotinic treatment that causes this augmented response. Stimulation of chromaffin cells with nicotine results in disassembly of the cortical actin network (Cheek and Burgoyne, 1986). The actin disassembly was not seen with other secretagogues including high K^+. Thus it was conceivable that nicotine pretreatment might 'open' the cytoskeletal network, allowing more granules access to the plasma membrane upon treatment with

Fig 7.3A Response of cells to metabolic inhibition following pretreatment with nicotine. Cells were treated with Locke's buffer plus 10μM nicotine; after 10min this buffer was replaced with Locke's buffer (Basal); Locke's buffer plus 10μM nicotine or Locke's buffer plus metabolic inhibitors (M.I.). Catecholamine release was measured after a further 10min. The figure shows the response in untreated cells (Normal response) and after 10min nicotine exposure (Nic pretreated). Results are expressed as percentage catecholamine release of total cell catecholamine content at the start of the experiment, means ± SE of triplicate determinations.

Fig 7.3B Release of catecholamines with metabolic inhibitors following pretreatment with nicotine for various times. Cells were treated with Locke's buffer plus 10μM nicotine for different lengths of time from 0.5 to 10min. The buffer was then replaced with Locke's buffer (basal release) or Locke's buffer plus metabolic inhibitors. Catecholamine release was measured after 10min and was expressed as a percentage release of total cell catecholamine content at the start of the experiment in response to metabolic inhibitors after subtraction of basal release. This is plotted as a function of the time of nicotine pretreatment. Results are means ± SE of triplicate determinations.


Normal response
Nic pretreated
A
% Catecholamine release
0
2
4
6
8
10
12
Basal
10μM Nicotine
M.I.
B
% Catecholamine release above basal
0
2
4
6
8
10
Time (min)

metabolic inhibitors resulting in a second larger release of catecholamines. This was investigated in more detail by pretreating the cells with 10μM nicotine for increasing lengths of time, ranging from 0.5 to 10min, and then measuring the catecholamine release produced by subsequently replacing the nicotine with metabolic inhibitors for 10min. Basal release values (10min with Locke's buffer alone following nicotine pretreatment) were subtracted. The results are shown in Fig 7.3B.

Treatment of this batch of cells with metabolic inhibitors alone produced a release of 4.3% above basal levels. Following nicotine pretreatment a release of up to 10% above background was observed. However, no release above that obtained in the absence of pretreatment was seen until after 1 to 2min pretreatment with nicotine. Consequently, this potentiation of the response cannot be explained by actin disassembly, since disassembly is followed by reassembly within the first 60s after nicotine stimulation (Cheek and Burgoyne, 1986). Furthermore, a recent report has demonstrated that high K^+ also elicits a reduction in filamentous actin at the cell periphery (Burgoyne *et al.*, 1989). Therefore, the nicotine-evoked potentiation of the response to metabolic inhibitors must either be due to some event at the nicotinic receptor itself or be due to some event subsequent to actin disassembly that is not triggered by high K^+.

7.3 Discussion

In order to gain further insights into the mechanism of catecholamine release evoked by metabolic inhibition the role of intracellular cytosolic free Ca^{2+} was examined using the fluorescent Ca^{2+} indicator Fura-2. Both the free and Ca^{2+}-bound forms fluoresce strongly but their excitation peaks are at different wavelengths. Thus by measuring the ratio of Ca^{2+}-bound to free dye it is possible to estimate cytosolic free Ca^{2+}. This method overcomes the problems of dye concentration and cell thickness encountered with single wavelength measurements. One problem encountered in measuring the fluorescence of a cell population in suspension was a gradual increase in apparent basal levels with time after loading. This is in fact due to some leakage of Fura-2 from intact cells despite being kept to a minimum by keeping the cells on ice. As the experiments involved monitoring changes in $[Ca^{2+}]_i$ rather than determination of absolute values, this problem was not considered to be critical to the results obtained.

The secretagogues nicotine, acetylcholine, and high K^+ all showed a similar

transient response that was dependent on extracellular Ca^{2+}, as reported by other workers (Burgoyne and Cheek, 1985). In contrast, metabolic inhibition caused a large rise in $[Ca^{2+}]_i$ that was retained for at least 5min. The $[Ca^{2+}]_i$ rise was relatively slow compared with the rise evoked by other secretagogues. In single cells the rise in response to nicotine occurs within 1s and the maximal response is reached after 27s (O' Sullivan *et al.*, 1989). The rise induced by metabolic inhibition did not reach a plateau until several minutes after the initial rise was observed.

As catecholamine release occurred independently of external calcium it was postulated that this was due to a mobilisation of internal Ca^{2+}. In the absence of extracellular Ca^{2+} there was a rise in $[Ca^{2+}]_i$ but this was much smaller than that seen in the presence of Ca^{2+}. Muscarinic secretagogues mobilise an IP_3 sensitive Ca^{2+} store independently of external Ca^{2+}. The subsequent rise in $[Ca^{2+}]_i$ does not, however, evoke catecholamine secretion (O' Sullivan and Burgoyne, 1989). This lack of secretion has been attributed to the intracellular localisation of the Ca^{2+}, this being at one pole of the cell away from the secretory granules (O' Sullivan *et al.*, 1989). Agents such as bradykinin which do evoke a small secretory response also mobilise calcium release from an IP_3 sensitive store but it now appears to be a secondary influx of Ca^{2+} at one pole of the cell rather than the released internal Ca^{2+} that is the trigger for exocytosis (Cheek *et al.*, 1989a).

It is not possible to discern from these results whether or not it is the rise in $[Ca^{2+}]_i$ evoked by metabolic inhibition that causes catecholamine release. Recent studies on the ability of nicotine stimulation to evoke secretion compared with muscarinic stimulation suggest that it is the Ca^{2+} influx rather than mobilisation of internal Ca^{2+} that is the trigger for exocytosis. The localisation of Ca^{2+} also appears to be a critical factor in exocytosis (Cheek *et al.*, 1989a; Cheek *et al.*, 1989b; and O' Sullivan *et al.*, 1989).

The small increase in $[Ca^{2+}]_i$ caused by metabolic inhibition in the absence of extracellular Ca^{2+} may not be sufficient to trigger secretion. However, only a small rise in $[Ca^{2+}]_i$ around the periphery of the cell just beneath the plasma membrane is sufficient to evoke exocytosis in response to nicotine stimulation (O'

Sullivan *et al.*, 1989); the large rise in $[Ca^{2+}]_i$ across the whole of the cell occurs after catecholamine secretion has been initiated. It is conceivable that the small rise in $[Ca^{2+}]_i$ may be localised in the region of the cell close to the granules and so elicit secretion. It is also plausible that metabolic inhibition may alter the calcium sensitivity of the secretory mechanism so that exocytosis can be triggered even at near resting $[Ca^{2+}]_i$ and that this rise in $[Ca^{2+}]_i$ can be achieved independently of extracellular Ca^{2+}. Phorbol esters, for example, increase the affinity of exocytosis for Ca^{2+} in platelets so that secretion of serotonin takes place at or near the resting $[Ca^{2+}]_i$ of the cell. Phorbol esters are able to substitute for DAG and activate protein kinase C (Knight, 1987).

Such a mechanism could also explain the potentiation of the response to metabolic inhibitors induced by nicotine. Following nicotine stimulation protein kinase C is translocated to the plasma membrane (Terbush and Holz,1986). Nicotine stimulation produces a reduction in filamentous actin and is able to generate inositol phosphates and DAG in the presence or absence of external Ca^{2+} (Burgoyne *et al.*, 1989). Depolarisation with high K^+ also results in a reduction in filamentous actin but only about half that evoked by nicotine. In addition, high K^+ activates phospholipase C to generate inositol phosphates and DAG but both this response and the reduction of filamentous actin are abolished in the absence of external Ca^{2+} (Burgoyne *et al.*, 1989). This is consistent with previous reports where nicotine but not high K^+ caused disassembly of cytoskeletal actin (Cheek and Burgoyne, 1986). Pretreatment of cells with nicotine would activate phospholipase C and the ensuing secretory events could leave the cells in a 'primed' state so that the subsequent rise in $[Ca^{2+}]_i$ evoked by metabolic inhibition may be sufficient to trigger exocytosis. Nicotinic stimulation must activate mechanisms additional to those controlling high K^+ stimulated release since only the former secretagogue potentiates the response to metabolic inhibition. This may involve elements of the cytoskeletal network.

At present, however, such a mechanism is only hypothetical. The use of specific inhibitors to identify the internal Ca^{2+} store responsible for the rise in $[Ca^{2+}]_i$ seen in the absence of extracellular Ca^{2+} may provide further insights into the release mechanism.

Chapter Eight

Discussion and Conclusions

8.1 Chromaffin cells and anoxia

Isolated bovine adrenal chromaffin cells have been utilised to study catecholamine release under conditions of anoxia and metabolic inhibition. In the perfused rat heart such conditions evoke a large overflow of noradrenaline from the sympathetic nerve terminals by a putative mechanism of carrier-mediated efflux (Schömig *et al.*, 1984). This overflow only becomes significant in regions of the heart that are severely ischaemic. In bordering regions where ischaemia is less severe there may be sufficient glycolytic activity to maintain sympathetic nerve function and to keep extracellular noradrenaline levels low by active re-uptake, despite increased sympathetic activity. This heterogeneity of response is in itself a major factor in triggering the onset of arrhythmias.

Ca^{2+}-independence and inhibition with $uptake_1$ blockers were the criteria used to identify the mechanism of carrier-mediated efflux in isolated perfused rat hearts (Schömig *et al.*, 1984). Conditions designed to mimic ischaemia in isolated chromaffin cells (both anoxia and metabolic inhibition) were found to evoke a release of catecholamines that was independent of external Ca^{2+}. This release, however, was not inhibited by $uptake_1$ blockers. Furthermore, catecholamines were co-released with the soluble granule protein chromogranin A and with ATP; there was no release of the cytosolic protein LDH, indicating that release occurred by exocytosis. Closer investigation of this catecholamine release revealed that it was accompanied by a significant uptake of extracellular Na^+ and a rise in $[Ca^{2+}]_i$.

The rise in $[Ca^{2+}]_i$ was mainly due to in flux of external Ca^{2+}. There was also a small rise in internal Ca^{2+} seen in the absence of external Ca^{2+} and this was attributed to mobilisation of internal Ca^{2+} stores. The catecholamine release appeared to be dependent on extracellular Na^+, but the role of this ion in the mechanism of release remains unclear.

8.2 Chromaffin cells as a model for sympathetic neurons

Chromaffin cells were originally used as a model for sympathetic neurons as they exhibit many similar biochemical and morphological features and share the same embryonic origin, deriving from the neural crest (Winkler, 1988). Observations in this study and by others, however, have illustrated distinct differences between the two cell types which indicate that they are not directly comparable.

Foetal chromaffin cells in culture will differentiate into sympathetic neurons

rather than chromaffin cells unless supplied with exogenous glucocorticoids (Anderson, 1989). Adult chromaffin cells, however, will not revert to sympathetic neurons unless they are supplemented with nerve growth factor, which results in neurite outgrowth (Anderson, 1989). Comparative studies on PC12 cells and chromaffin cells have suggested that PC12 cells may be closer biochemically and functionally to sympathetic neurons than chromaffin cells. For instance, sympathetic neurons and PC12 cells both possess MAO type A compared with chromaffin cells that possess primarily MAO type B (Youdim *et al.*, 1984). In addition, PC12 cells, like neurons, possess a tyramine releasable pool of catecholamines unlike chromaffin cells which show no response to prolonged exposure to tyramine (Youdim *et al.*, 1986; and Chapter 5).

The difference in the response of chromaffin cells to conditions of metabolic inhibition compared with cardiac sympathetic neurons led to an examination of carrier-reversible catecholamine release. This mechanism was first identified in sympathetic neurons from the rat vas deferens (Paton, 1976) and has subsequently been demonstrated in PC12 cells (Bönisch *et al.*, 1984). It became apparent, however, that even under artificial conditions designed to elicit and to demonstrate carrier-reversal, there was no increased efflux of catecholamines from chromaffin cells (Chapter 5; and Powis *et al.*, 1989). It is therefore debatable whether the uptake carrier of mature chromaffin cells does in fact operate in reverse; in contrast to that in neurons it may be 'gated' in some way.

8.3 Catecholamine release in neonates

While the above observations discounted carrier-mediated efflux as a mechanism of release from chromaffin cells in anoxia, there are indications that the exocytotic release evoked by metabolic inhibition may be of physiological importance in neonates.

In human neonates there is a very large surge in circulating catecholamines during parturition in response to hypoxia (Lagercrantz and Slotkin, 1986). Studies on animal models have demonstrated that this release is critical in protecting the neonate against hypoxia and asphyxia; and also in assisting it in adapting cardiovascular and respiratory function for survival outside the womb. In many mammalian species, including man, sympathetic innervation is absent or non-functional at birth. Consequently the surge in catecholamines must be the result of a direct effect of hypoxia in the adrenal medulla (Slotkin and Seidler, 1988). Neonatal rat adrenal glands *in vitro* show a full response to direct

stimulation with nicotine or high K^+ even at 2 days old, indicating that the nicotinic receptors are functional and that the full exocytotic mechanism is operative even though the chromaffin cells *in situ* are non-innervated (Slotkin and Seidler, 1988).

These observations (for discussion see Slotkin and Seidler, 1988) have raised the question of whether hypoxia causes a loss of catecholamine storage capacity and subsequent leakage of catecholamines into the cytoplasm and hence out of the cells, or whether it can directly trigger exocytosis. This was investigated in the neonatal rat by Seidler and Slotkin (1986). By examining the effects of hypoxia and nicotine on catecholamine levels and on uptake capabilities they concluded that hypoxic catecholamine release was proceeded by a mechanism of exocytosis. Hypoxia- and nicotine-induced depletion of catecholamines were both accompanied by an equivalent reduction in uptake indicating loss of granule integrity, which is characteristic of exocytosis. It was postulated that the stimulus for this release may either be an influx of Ca^{2+} through voltage-dependent channels or a release of internal Ca^{2+} from intracellular stores such as the mitochondria; both responses being mediated by hypoxia. In addition, similar observations have been described in chromaffin cells isolated from foetal sheep (Cheung, 1989). Hypoxia elicited a direct response of isolated cells which did not decrease with maturation; this is in contrast to the response seen when release elicited by hypoxia declines with age in parallel with the development of innervation(Slotkin and Seidler, 1988). Upon denervation, however, the ability of the adrenal gland to respond to hypoxia is restored.

Consequently, using isolated chromaffin cells from either an adult or neonate may give responses analogous to those of neonatal cells *in vivo*. The catecholamine release discussed in the present study may thus be comparable to this non-neurogenic response.

8.4 The role of calcium

The key difference between this anoxia-induced catecholamine release and the normal exocytotic response to external stimuli is the former's independence of external Ca^{2+}. This does not, however, imply a total Ca^{2+}-independence. Using the fluorescent Ca^{2+}-indicator Fura-2 a rise in $[Ca^{2+}]_i$ was identified in the absence of external Ca^{2+}. This must be induced by the anoxic conditions and is presumably sufficient in size and cellular location to trigger catecholamine release (Chapter

7). The source of this Ca^{2+} is unknown. It could, for example, be mitochondrial, since anoxic incubation of isolated rat liver mitochondria leads to ATP depletion and release of Ca^{2+}(Nishida *et al.*, 1989) over a few minutes. This Ca^{2+} efflux is associated with uncoupling of the mitochondria through activation of phospholipase A_2 and disruption of the proton impermeability of the inner mitochondrial membrane.

The question of Ca^{2+}-independent exocytosis has recently been considered in depth by Knight *et al.* (1989). They concluded that the mechanism of exocytosis is Ca^{2+}-dependent. DAG production increases the Ca^{2+} sensitivity of the secretory process so in some cases exocytosis can be triggered at or below the basal $[Ca^{2+}]_i$. In other cases the extent of catecholamine release is increased rather than the Ca^{2+} affinity. In chromaffin cells a small rise in $[Ca^{2+}]_i$ just beneath the plasma membrane appears to be sufficient to trigger secretion (O'Sullivan *et al.*, 1989).

In addition, Ca^{2+}-dependent secretion is affected by various modulators which act either by increasing the Ca^{2+} affinity of the secretory process or by increasing the extent of release. For instance, the phorbol ester TPA causes an increase in Ca^{2+} affinity so that the Ca^{2+} threshold for secretion in electropermeabilised chromaffin cells is lowered (Knight *et al.*, 1989).

The most attractive mechanism of release would thus seem to be one where conditions of metabolic inhibition increase the Ca^{2+} sensitivity of the secretory process so that release is triggered by a small rise in $[Ca^{2+}]_i$. This could be investigated by examining the effect of metabolic inhibitors on the Ca^{2+} activation curve in permeabilised chromaffin cells in experiments similar to those performed by Knight (1987) with TPA.

In addition, chromaffin granules are translocated to the plasma membrane independently of external Ca^{2+} (Burgoyne *et al.*, 1982) and the metabolic changes caused by anoxia or metabolic inhibition may be sufficient to trigger secretion from those granules that are close to the plasma membrane. It would thus be interesting to investigate whether anoxia can lead to slow rearrangements of the peripheral cytoskeleton, such as can be induced by Ca^{2+} alone in permeabilised cells (Burgoyne *et al.*, 1989).

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